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Regulation of nitrate reduction in wheat leaves

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Abstract. Wheat leaves exposed to 710 nm monochromatic light, when only photosystem 1 operates, reduced small but significant amount of nitrate to nitrite. This could be due to partial inhibition of mitochondrial oxidation of NADH, brought about by cyclic photophosphorylation. Under dark aerobic conditions, citric acid cycle intermediates only slightly stimulated nitrate reduction. Under dark anaerobic conditions, when maximum reduction of nitrate occurred, the time course showed a 1:1 stoichiometry between nitrite and CO_2. It is suggested that for maximum reduction of nitrate under physiological conditions, CO_2 fixation and export of ATP via triose phosphate shuttle is essential.

Keywords. Nitrate reduction; photosystems; 710 nm light; CO_2 evolution.

Introduction

In plants, nitrate reductase (E.C. 1.6.6.1) is generally believed to be located in the cytoplasm of green cells (Rathnam and Das, 1974). Although it has also been suggested that the enzyme may be loosely associated with the outer surface of the chloroplast membrane, it is agreed by most workers that NADH produced in or supplied to the cytoplasm is used as a reductant for the reduction of nitrate to nitrite (Butz and Jackson, 1977). Nitrite formed is translocated to the chloroplasts and assimilated to form ammonia and glutamate by the combined action of nitrite reductase (E.C. 1.7.7.1) glutamine synthetase (E.C. 6.3.1.2) and glutamate synthase (E.C. 2.6.1.53). These three enzymes are dependent on light reactions of photosynthesis for the supply of reductants, reduced ferredoxin and ATP (Miflin and Lea, 1977). Canvin and Atkins (1974) showed that the activity of nitrate reductase is also light dependent and ceases abruptly when light is extinguished Sawhney *et al.* (1978 a, b) showed that light regulates nitrate reduction by inhibiting mitochondrial oxidation of NADH, brought about by enhanced cytoplasmic adenylate energy charge *via* photophosphorylation and export of triose phosphates from the chloroplasts (Heber 1974, Gierch *et al.,* 1979). NADH thus formed is then available for nitrate reductase. Subbalakshmi *et al.* (1979) suggested that generation of NADH in the mitochondria and prevention of its oxidation by O_2 are the factors that regulate nitrate reduction in green leaves. Rama Rao *et al.* (1981) and Naik and Nicholas (1981) showed a close relationship between nitrate reduc-

tion and CO_2 evolution under anaerobic conditions in wheat leaves. In this paper we report the reduction of nitrate under aerobic conditions in 710 nm light and in darkness and on the time course of nitrate reduction and CO_2 evolution under anaerobic conditions.

Materials and methods

Ten-day-old wheat seedlings (cv-Halbredt, grown in a phytotron (16 h light, 8 h dark) in vermiculite with 10 mM KNO_3 were used.

In vivo dark anaerobic assay of nitrate reduction

Wheat leaves were cut into small discs (0.5 cm) and 100 mg tissue was placed in a Thunberg tube (25 ml). After thorough evacuation with an Edwards evacuating pump, the Thunberg tubes were closed and incubated in the dark at 25°C. The leaves were extracted with boiling water and nitrite determined as described previously (Sawhney *et al.*, 1978a). For the infiltration of organic acids into the leaf tissue, leaf discs were suspended at pH 5.0 (table 2). The acid pH facilitated the penetration of organic acids (Sawhney *et al.*, 1978b).

Nitrite reduction in 710 nm light

In experiments involving monochromatic light, a Philips 500 watt flood lamp fitted with interference filters to give 710 nm light was used as described by Jordan and Givan (1979). The irradiance at the level of the leaf discs was 20.7 w m². Wheat leaf discs from 10-day-old seedlings grown with 10 mM KNO_3 were exposed to the light for 15 min and nitrite formed was determined as described previously (Sawhney *et al.*, 1978a). In order to prevent any rise in temperature due to exposure to far red light a petri dish with a thin layer of water was placed between the light source and the leaf discs. A small table fan was also operated to blow off the hot air.

$^{14}CO_2$ evolution from endogenously-labelled substrates

Leaves obtained from plants grown with and without nitrate were placed in a closed chamber and exposed to $^{14}CO_2$ in the dark as described earlier (Naik and Nicholas 1981). After 1 h incubation at 25°, the total $^{14}CO_2$ assimilated was determined by extracting a sample of the leaves with hot 70% v/v ethanol (Jordan and Givan 1979). Samples of the remaining leaf material (100 mg) were incubated in Thunberg tubes under dark anaerobic conditions at 25°C for different time intervals. A small filter paper disc (1 cm²) soaked in 20 µl of 20% KOH was suspended from the lid of the tube by means of a bent pin. At intervals, different tubes were opened and $^{14}CO_2$ absorbed was determined in a Packard Tri-carb liquid scintillation spectrometer. Nitrite formed in the leaf discs was also determined as described above.

Time source of CO_2 evolution and NO_2^- production

Leaf discs of wheat (100 mg) from plant grown with nitrate were placed in Warburg flasks. Air was removed with a vacuum pump and the flasks were filled with a mixture of N_2 and argon gasses (95 and 5% v/v respectively). The leaf discs were then incubated for various time intervals in the dark at 25°C. The CO_2 released was monitored in a mass spectrometer, as described by Naik and Nicholas (1981).

with argon (40) as an internal standard. The nitrite formed was determined in boiling water extracts of leaf discs as described previously (Sawhney *et al.*, 1978a).

Results

Effect of 710 nm monochromatic light

Since reduced ferredoxin is the physiological electron donor for nitrite reductase in the chloroplasts, operation of photosystems 1 and 2 is essential for the reduction of nitrite to ammonia (Miflin and Lea, 1977). In 710 nm light, when only photosystem 1 operates, nitrite can not be reduced. ATP is derived from cyclic photophosphorylation under these conditions and if there is a significant transfer of ATP to the cytoplasm, sufficient to inhibit the oxidation of NADH in the mitochondria, it might be possible to detect reduction of nitrate to nitrite under aerobic conditions. Results presented in table 1 show that significant amount of nitrite

Table 1. Reduction of nitrate in wheat leaves exposed to 710 nm light.

Time (min)	Dark		Light (710 nm)
	Anaerobic	Aerobic	Aerobic
	nmol NO_2^- formed/g fresh wt.		
5	370	nil	16
10	540	nil	33
15	1750	nil	100
20	2500	nil	150

Leaves from 10 day old seedlings, containing sufficient endogenous nitrate were incubated under anaerobic conditions or in air in 710 nm light and nitrite formed was determined as described in Materials and Methods.

accumulated in wheat leaf discs exposed to 710 nm light. The reaction was however, extremely slow as compared with the anaerobic treatment, when mitochondrial oxidation of NADH is completely inhibited. Rama Rao *et al.* (1981) reported that under aerobic conditions in the dark nitrite is further reduced in leaves. However, this reaction commenced only after a time lag of 20 min. In the present experiment (table 1) the incubation was restricted to only 15 min in order to avoid any possible reduction of nitrite produced.

Effect of organic acids

Since competition for reducing equivalents from NADH, between O_2 and nitrate, regulates nitrate reduction, it is possible that under dark aerobic conditions, supply of NADH generating citric acid cyclic intermediates, may stimulate nitrate reduction. However, results described in table 2 show that as compared with the anaerobic control, the amount of nitrite formed in different treatments is extremely small. In the aerobic control also a small amount of nitrite was detected (60 nmol) while no nitrite was detected in leaf discs directly exposed to air in a

gaseous phase as shown in table 1. This eould be due to submergence of leaf discs in a liquid medium, which may impede the penetration of O_2 in the leaf discs to some extent (table 2). As compared to this control, glutamate, pyruvate and

Table 2. Effect of organic acids on nitrate reduction under dark aerobic conditions.

Concentration of substrates	2 Oxo-glutarate	Pyruvate	Succinate	Malate	Glutamate
	nmol NO_2^- formed/15 min/g fresh wt.				
25	70	105	120	75	120
50	60	120	150	90	160
100	25	165	95	90	180

Wheat leaf discs (100 mg) were suspended in 2 ml 0.1 M phosphate buffer pH 5.0, containing 10 mM KNO_3 and different substrates. After incubation at 25°C for 15 min in a reciprocating water bath for aeration, nitrite formed was determined as in Table 1. In the aerobic and anaerobic control experiments, 60 and 2000 nmol of NO_2^- was formed/15 min/g fresh wt. of leaf disk.

succinate stimulated nitrate reduction. L-Malate was less effective while, 2-oxoglutarate was inhibitory, probably because in its presence endogenous aspartate would generate oxaloacetate by transamination. In the presence of oxaloacetate, the malate dehydrogenase would oxidise NADH produced, for the synthesis of malate, because the equilibrium of this enzyme reaction favours malate synthesis.

Nitrite formation and CO_2 evolution under anaerobic conditions

Wheat leaves were initially exposed to $^{14}CO_2$ in the dark so that ^{14}C is not incorporated into sugars because NADPH and ATP are not produced in the dark. Under these conditions organic acids are exclusively labelled due to dark carboxylation reactions. When these leaf discs were subsequently incubated under dark anaerobic condition, $^{14}CO_2$ evolution depended on nitrate reduction as shown in table 3. In leaves lacking nitrate reductase, $^{14}CO_2$ evolution did not increase with the time of incubation. The time course of stoichiometry of CO_2 evolution to nitrite formed upto 75 min revealed a 1:1 relationship (table 4).

Discussion

Rama Rao *et al.* (1981) and Naik and Nicholas (1981) had suggested that NADH required for nitrate reductase is probably of mitochondrial origin and CO_2 evolution under anaerobic condition depends upon nitrate reduction and vice versa. These suggestions are confirmed by the results presented in table 3 and 4. The 1:1 stoichiometry at all points of time between nitrite and CO_2 formed suggests that citric acid cycle dehydrogenases which generate CO_2, simultaneously supply NADH for the activity of nitrate reductase. The export of NADH generated within the mitochondria can be via dicarboxylate malate: oxaloacetate shuttle as

Table 3. Time course of $^{14}CO_2$ evolution during anaerobic nitrate reduction in wheat leaf discs.

	−NO$_3$ plants		+NO$_3$ plants	
Time (m)	$^{14}CO_2$ cpm/g fresh wt.	NO_2^- n mol/g fresh wt.	$^{14}CO_2$ cpm/g fresh wt.	NO_2^- n mol/g fresh wt.
15	511	nil	1795	950
30	714	nil	2655	1600
45	520	nil	3553	2050
60	741	nil	3807	2560

Total $^{14}CO_2$ incorporated in wheat leaves during dark fixation with and without nitrate were 104,000 and 148,400 cpm/60 min/g fresh wt.

Leaves from wheat seedlings grown with or without nitrate were initially allowed to fix $^{14}CO_2$ in the dark for 1 h. Discs from these leaves were incubated under anaerobic conditions and $^{14}CO_2$ evolved and nitrite formed were measured as described in Materials and Methods.

Table 4. Time course of CO_2 evolution and nitrite formation

Expt. No	NO_2^-	CO_2	NO_2^-/CO_2
1.	325[a]	334[a]	0.97
2.	375	346	1.08
3.	337	351	0.96
	Time course		
Incubation time min.			
30	165	140	0.85
45	286	250	0.87
60	503	480	0.95
75	608	580	0.96

[a] nmol NO_2^- or CO_2 formed/h/100 mg fresh wt.

proposed by Woo *et al.* (1980) or via transmembrane transhydrogenase present in plant mitochondria which enables the dehydrogenases to reduce external NAD^+, as proposed by Day and Wiskich (1978).

In the presence of air a slight accumulation of nitrite was promoted when citric acid cycle intermediates were supplied to generate NADH (table 2). However, in the absence of inhibition of mitochondrial oxidation of NADH, the amount of

nitrite formed was extremely small. Under normal physiological conditions active photosynthesis, mitochondrial electron transfer process is most probably inhibited by the enhanced cytoplasmic adenylate energy charge, as explained earlier. Under these conditions, although nitrate is reduced rapidly, free nitrite cannot be detected because it is further assimilated in the chloroplast, as fast as it formed. In 710 nm light however, when only photosystem I operates, small but significant accumulation of nitrite is detected (table 1). In the absence of photo system 2, non cyclic photophosphorylation and synthesis of triose-phosphates are inhibited and hence export of ATP from the chloroplast via dihydrooxycetone phosphate: 3-phosphoglycerate shuttle cannot take place. However, according to Jordan and Givan (1979), ATP derived from cyclic photophosphorylation in 710 nm light may be transported to the cytosol and mitochondria, resulting in the partial inhibition of mitochondrial oxidation of NADH. They have suggested that cyclic photophosphorylation reactions can stimulate energy dependent processes outside the chloroplast, even in the absence of CO_2 assimilation and absence of photosystem-2 activity. Results in table 1 however suggest that for complete inhibition of mitochondrial oxidation of NADH, leading to the promotion of maximum reduction of nitrate, CO_2 fixation and subsequent export of ATP via the shuttle mechanisms may be essential.

Acknowledgements

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Prostaglandin synthetase activity of goat vesicular microsomes: Co-factor requirement and effect of calcium ions

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Abstract. The effects of several co-factors and bivalent cations on the activity of prostaglandin synthetase isolated from goat seminal vesicles were studied. Ca^{2+} appears to play a regulatory role in the biosynthesis of prostaglandin E_2 by goat vesicular microsomes as the normal parabolic time course of synthesis changed to a sigmoid curve in the presence of 4 mM Ca^{2+} and to nearly a hyperbolic pattern when the microsomes were preincubated with the metal ions. The Ca^{2+} modulated reaction showed increased rate of prostaglandin E_2 synthesis only when the period of incubation was extended beyond 30 min. The co-factor requirement of the goat enzyme was similar to that of the bovine and ovine prostaglandin synthetase systems.

Keywords. Prostaglandin synthetase; goat vesicular microsome; Ca^{2+} effect.

Introduction

Ovine (Bergstorm *et al.,* 1964; Wallach & Daniels, 1971; Nugteren *et al.,* 1966) and bovine (Takeguchi *et al.,* 1971; Flower *et al.,* 1973) seminal vesicles are known to be rich sources of prostaglandin synthetase. However, tissues of these animals are difficult to obtain for experimental purposes in many parts of India. We have therefore investigated an alternative source, namely the goat seminal vesicles, for the prostaglandin synthetase and determined the effect of various co-factors on the enzyme activity. It has been found that an active prostaglandin synthetase enzyme system is associated with the microsomal fraction of the goat vesicular gland. Although the co-factor requirement and pH profile of the goat prostaglandin synthetase are similar to those of the bovine or ovine systems, we would like to report here a regulatory role for Ca^{2+} in the biosynthesis of prostaglandin not previously investigated with the vesicular systems of other animals.

Materials and methods

Materials

Arachidonic acid and epinephrine bitartrate were purchased from Sigma Chemical Company, St. Louis, Missouri, USA and silica gel G, Type 60 from E. Merck,

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Abbreviations used: PGE_2, prostaglandin E_2; GSH, reduced glutathione.

Darmstadt, Germany. Fresh goat vesicular glands were collected from a local abattoir and kept frozen at-20°C. Standard prostaglandins were obtained from Upjohn Company, Kalamazoo, Michigan, USA as a gift. All other chemicals used were of the Analytical grade.

Preparation of microsomes

All operations were performed between 0-4°C. Frozen or fresh goat seminal vesicles were trimmed of excess fat and other tissues, diced and blended in a Waring Blendor for 2 min with two volumes of 0.1 M potassium phosphate buffer, pH 8.0. The homogenate was centrifuged for 10 min at 12,000 g in a Sorvall Model RC 5B centrifuge. The supernatant fraction was passed through a double layer of cheese cloth and centrifuged again at 100,000 g for 1 h in a Beckman Ultracentrifuge Model L5-50. The resulting pellet was suspended in 2 volumes of 0.1 M tris-HCl buffer, pH 8.2 and recentrifuged at 100,000 g for 1 h. The washed microsomal pellet was resuspended in 1 volume of 0.1 M tris-HCl buffer, pH 8.2 and stored at-20°C, distributed in several tubes. The protein content of microsomal fraction was 4-5 mg/g of wet tissue measured by Lowry's method (Lowry *et al.,* 1951).

Spectrophotometric assay of prostaglandin synthetase

The standard incubation mixture contained the following components per tube in a final volume of 0.5 ml: tris-HCl buffer, pH 8.2: 100 mM; arachidonic acid, 1 mM; epinephrine, 5 mM; reduced glutathione (GSH), 5 mM; and variable amount of microsomal fraction. For standard PGE_2 synthetase assay, incubations were done a final volume of 0.5 ml: tris-HCl buffer, pH 8.2, 100 mM; arachidonic acid, 1 mM; which was a little higher than the optimum, determined in a separate experiment (data not shown).

Arachidonic acid was added as an alcoholic solution but the final concentration of ethanol in the assay mixture was about 2%, which was without effect on the synthetase activity. Enzymatic reactions were initiated by the addition of micro-somes and terminated by the addition of 0.1 ml of 2 M HCl. In zero time control or blank tubes, microsomal fractions were added after acidification of the reaction mixture kept in an ice bath. To each tube, after incubation and acidification, 1.5 ml of ethyl acetate was added, mixed for 30 s using a vortex mixer and centrifuged for 3 min in a clinical centrifuge. Aliquots (1.0 ml) from the ethyl acetate layer were withdrawn and evaporated to dryness at room temperature under vacuum in small tubes. The prostaglandin E_2 content of each tube was then measured by a spectrophotometric assay based on a procedure described earlier (Yoshimoto *et al.,* 1970). After evaporation of the ethyl acetate, the products in each tube were mixed with 0.8 ml of methanol and 0.2 ml of 3 M KOH in 60% methanol to convert all PGE_2 to prostaglandin B_2. The tubes were kept at room temperature for 15 min and then the absorbance at 278 nm was measured. The PGE_2 content was calculated on the basis of a standard curve obtained in the same way but using known quantities of PGE_2 in the tubes.

The prostaglandin synthetase activity is expressed as nmol of PGE_2 formed/min/ mg of microsomal protein under the conditions indicated.

Thin-layer chromatography

Silica gel G plates of thickness, 0.5 mm, were used. The plates were developed up
to a distance of about 18 cm in the solvent system ethyl acetate:water:isooctane:
acetic acid (11:10:5:2, upper-phase). The prostaglandin spots were located by
spraying with 10% anisaldehyde in 10% ethanolic sulphuric acid and heating at
90°C for 10 min (Keifer *et al.,* 1975). By this method, prostaglandins are well
separated, each showing its characteristic coloured spot.

Results

Synthesis of PGE$_2$ by goat vesicular microsomes

The products of a preparative scale reaction mixture were tested by thin-layer
chromatography with authentic reference prostaglandins. The major product was
PGE$_2$ along with some PGF$_{2\alpha}$ and PGD$_2$. The R$_f$ values of these compounds in
ethyl acetate:water:isooctane:acetic acid solvent system were identical with those
of standards (figure 1).
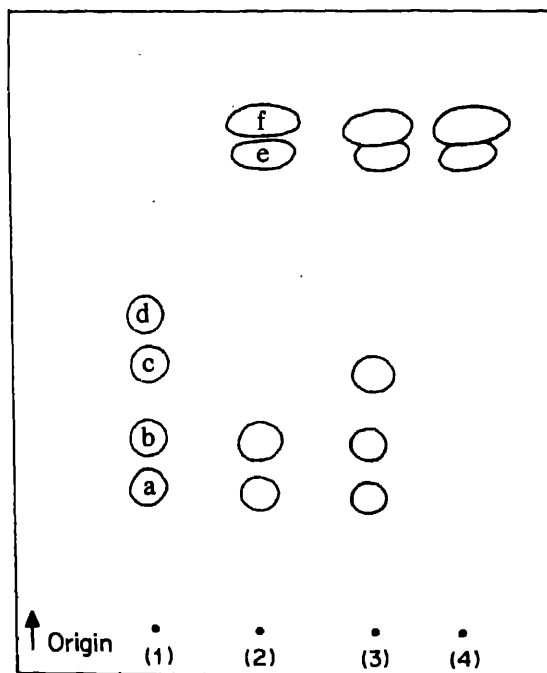
Figure 1. Thin-layer chromatogram of the products of oxygenation of arachidonic acid by the
prostaglandin synthetase system of goat vesicular microsomes. (1) Control, containing only
standard prostaglandin mixture, 0.5 nmol each of prostaglandin F$_{2\alpha}$ (a), prostaglandin E$_2$ (b),
prostaglandin D$_2$ (c), prostaglandin B$_2$ (d); spots (e) and (f) are unidentified product and
unreacted arachidonic acid respectively.

Effect of externally added cofactors

In order to determine the optimum conditions necessary for the assay of the enzyme activity associated with the goat vesicular microsomes by the spectrophotometric method, the effect of different externally added co-factors on the biosynthesis of PGE_2 was studied (table 1). As in bovine and ovine systems, GSH and

Table 1. Requirement of substrate and cofactors for the biosynthesis of prostaglandin E_2 by goat vesicular microsomes

Substrate and cofactors	PGE_2 formed (nmol/mg protein/min)
Complete system	18.1
—Arachidonic acid	0.58
—GSH	9.1
—Epinephrine	4.2
—GSH, — epinephrine	0.29
—Arachidonic acid, —GSH, — epinephrine	0.14
—GSH, — epinephrine, + ascorbic acid (5)	0.58
—GSH, + ascorbic acid (5)	11.3
—GSH, — epinephrine, +NADPH (0.2)	0.58
—GSH, +NADPH (0.2)	9.2

PGE_2 synthesized in 3 min at 37°C in different mixtures of substrate and cofactors by goat vesicular microsomal fraction were measured. The procedures for the preparation of microsomal fraction from goat vesicular gland, assay of its protein content and the spectrophtometric assay of PGE_2 formed were as described in the Materials and Methods section. The complete system contains 1 mM arachidonic acid, 5 mM GSH, 5 mM epinephrine, 100 mM tris-HCl (pH 8.2) and a microsomal fraction equivalent to 0.65 mg protein in a total volume of 0.5 ml; + or − denotes addition or omission of component(s) in the complete system; number within parenthesis denotes concentration (mM) of the component in the reaction mixture.

epinephrine in combinations were the most effective. Hydroquinone could replace epinephrine as a co-factor, but the former was not used because it produced high blank reading in the spectrophotometric assay. Neither NADPH nor ascorbic acid could replace GSH for PGE_2 biosynthesis. There was no requirement for added heme in the assay system; this was obviously due to the presence of enough bound-heme in the vesicular microsomal fractions (Van Der Ouderra *et al.*, 1977). The pH optimum for the enzyme was 8.2 (data not shown).

Even in the presence of co-factors required for the optimum PGE_2 biosynthesis by goat vesicular microsomes, the product formation decreased rapidly after a few min (figure 2) which was probably due to autoinactivation of the synthetase system (Smith and Lands, 1972); however, using a short time of incubation (3 min) a linear relationship between PGE_2 synthesized and the amount of microsomal protein added to catalyze the reaction could be shown.
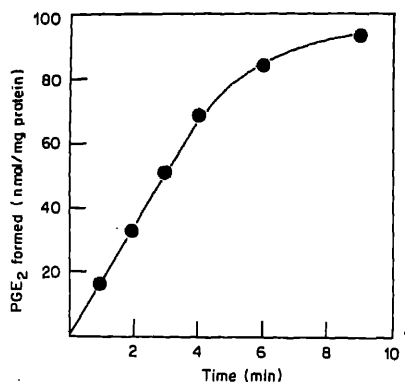
Figure 2. Effect of incubation time. All tubes contained the standard incubation mixtu\.\.

Effect of bivalent metal ions on PGE₂ biosynthesis

That prostaglandin synthetase activity of vesicular microsomes progressively diminishes with time during incubation is quite well-known, but the reason for this autoinactivation is not clear (Hemler & Lands, 1980). When the PGE_2 synthesis catalyzed by the goat vesicular microsomes at 37°C was plotted against time, a parabolic response curve was obtained as expected (figure 3a). Different compounds were checked for their possible role in the suppression of loss of prostaglandin synthetase activity with time, which was particularly pronounced at the later period of incubation. Among the ionic compounds tested, Ca^{2+} appeared to exert an effect on prostaglandin synthetase system that somehow prevented this loss of activity to a significant extent. The presence of Ca^{2+} appeared to stimulate the biosynthesis of PGE_2 by goat vesicular microsomes when
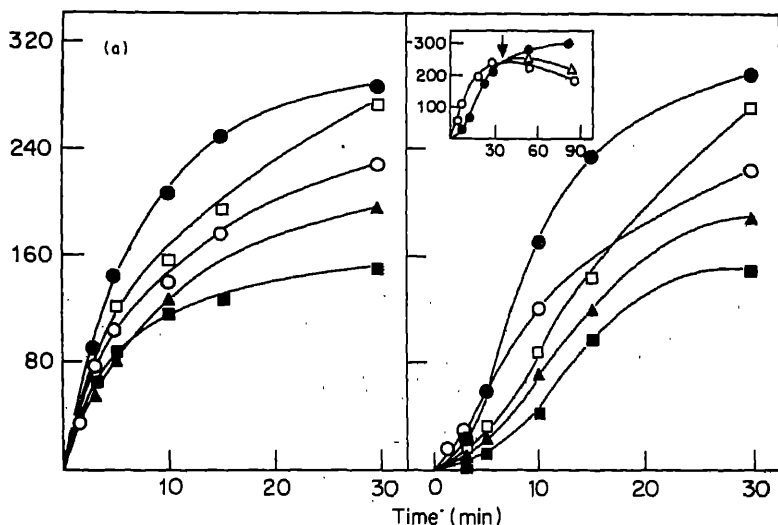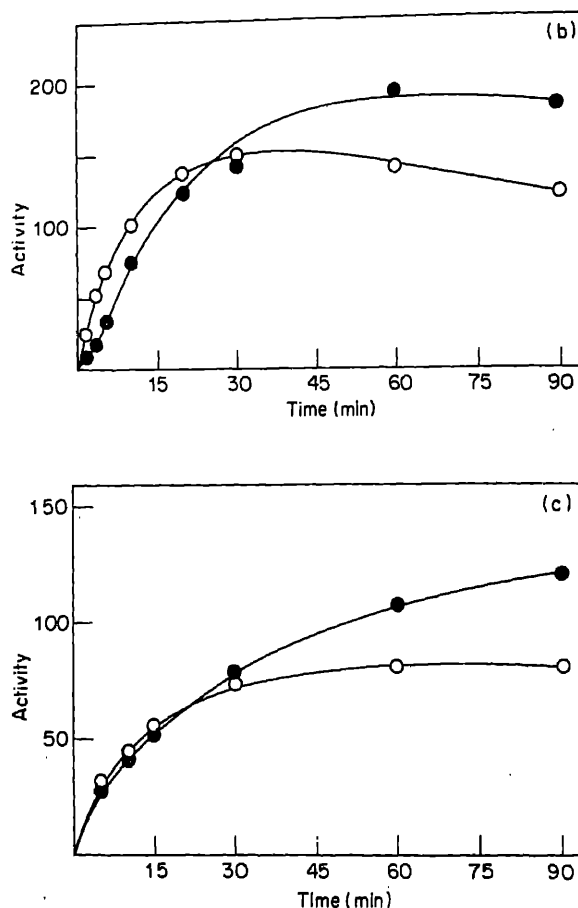
Figure 3. Effect of Ca^{2+} on the kinetics of prostaglandin E_2 biosynthesis. (a) The curves on the left represent the kinetics of PGE_2 production at $37°C$ upto 30 min, determined on different days with same microsomal preparation, using standard assay mixture without Ca^{2+}. Curves on the right with similar notations correspond to the same assays in presence of 4mM Ca^{2+}. In the inset the kinetics upto 90 min have been represented where notations are: (O) without Ca^{2+}; (●) with 4 mM Ca^{2+} added at 0 min, (Δ) with 4 mM Ca^{2+} added at 30 min. (b) and (c). The standard incubation mixture without cofactors (glutathione and epinephrine) were preincubated at $0°C$ (b) and $30°C$ (c) for 30 min with (●) and without (O) 4 mM Ca^{2+} (0 time control). Subsequent incubations were carried out at $37°C$ for 90 min after adding the cofactors to start the reaction. Activity=nmol of PGE_2 formed/mg protein.

the reaction time allowed was 1 h, and it distinctly showed a maximum in the region of 4 mM Ca^{2+} (figure 4). Effect of a number of bivalent metal ions was therefore tested on PGE_2 biosynthesis allowing 30 min, 60 min and 90 min incubation periods. As can be seen in table 2, Sr^{2+} shows an effect similar to Ca^{2+} but somewhat less pronounced, whereas Mn^{2+} exerts only an inhibitory effect; Mg^{2+} failed to show any effect at all. In addition, it is also clear that the stimulation of PGE_2 synthesis
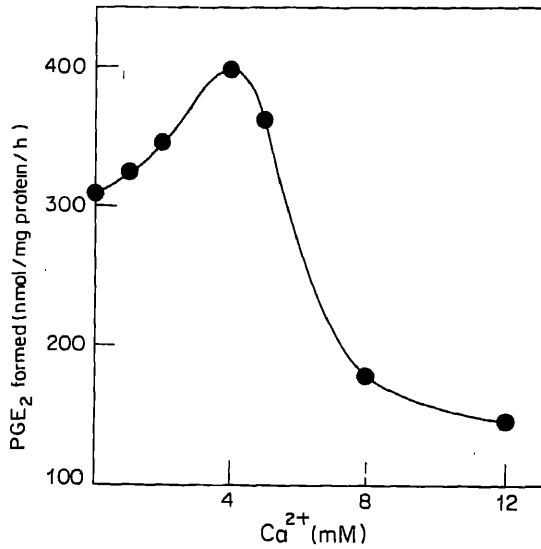
Figure 4. Effect of Ca²⁺ concentration on prostaglandin E₂ synthesis by microsomal fraction. To the standard incubation mixture increasing amounts of Ca²⁺ were added and incubated for 60 min.

by Ca^{2+} or Sr^{2+} can be seen only on long term incubation (60 min or 90 min) with the microsomes, and this stimulatory effect is hardly noticeable at 30 min.

Table 2. Effect of bivalent metal ion on prostaglandin E₂ biosynthesis by goat vesicular microsome

Metal ions added	PGE₂ formed (nmol/mg protein)		
	30 min	60 min	90 min
Standard incubation mixture	218.8	216.0	203.5
+Ca²⁺	216.9	314.9	281.0
+Sr²⁺	201.8	282.9	262.1
+Mg²⁺	199.5	201.5	203.5
+Mn²⁺	105.5	107.5	98.0

PGE₂ formed by goat vesicular microsomal fraction, in a standard incubation mixture in presence of different bivalent metal ions was measured. The standard incubation mixture contained microsomal fraction equivalent to 0.65 mg protein and 4 mM bivalent metal ion when added.

Effect of Ca^{2+} on the kinetics of PGE_2 biosynthesis

To understand the nature of the effect of Ca^{2+} on the microsomal prostaglandin synthetase system, we measured the formation of PGE_2 over a long period of time in the presence and absence of 4 mM Ca^{2+}. The results are shown in figure 3a. When the standard incubation mixture (1 mM arachidonic acid) was used, the plot of PGE_2 formed vs time was parabolic and this changed to a sigmoidal shape on the additional of 4 mM Ca^{2+}. PGE_2 synthesis showed an initial lag in the presence of Ca^{2+} which was absent in the control (i.e. without Ca^{2+}), but after about 30 min the synthesis in the former caught up with the latter and both increasing to different maxima; the maximum obtained in the presence of Ca^{2+} was higher. Figure 3a (inset) also shows that when the addition of 4 mM Ca^{2+} to the standard incubation mixture was made after 30 min of enzyme incubation at 37°C, there was no significant alteration of the kinetic curve for the next 60 min. It shows that a pre-incubation with Ca^{2+} is necessary for the microsomal prostaglandin synthetase system to prolong the maximal activity. Furthermore, assay of the same preparation for enzyme activity, as represented by the different curves in figure 3a, at different days of storage indicate loss of activity but all of them showing the same sigmoidal kinetics.

Preincubation of the enzyme system with Ca^{2+} at 0°C (with arachidonic acid but without co-factors in order to prevent PGE_2 synthesis during preincubation) for 30 min did not alter the sigmoid nature of the PGE_2 synthesis vs time curve (figure 3b) but it changed to hyperbolic shape when the same preincubation was carried out at 30°C (figure 3c). The microsomes preincubated with Ca^{2+} at 30°C for 30 min in the incubation mixture without the co-factors, therefore, showed no initial lag of PGE_2 synthesis when the reaction was started with the co-factors but it did show a higher maximum. Obviously the modulation of the prostaglandin synthetase system induced by Ca^{2+} is an energy dependent process but its mechanism remains unknown. There was, however, a significant loss of enzyme activity during the preincubation, particularly so at 30°C, (compare the enzyme activity in figure 3a, 3b and 3c) as the washed microsomal enzyme is quite unstable. The loss of activity during preincubation was still higher in the absence of arachidonic acid, and therefore, arachidonic acid was included in all preincubation mixtures. This requirement of arachidonic acid may be related to the hydroperoxide (which may be produced during preincubation) activation of the enzyme which eliminates the initial lag period (Hemler & Lands, 1980).

Modulation of the PGE_2 synthesis activity of the vesicular microsomes by Ca^{2+} can be shown more dramatically when the rate of PGE_2 synthesis is plotted against time (figure 5). In the control system (standard incubation mixture) the rate of PGE_2 synthesis decreases markedly almost from the very beginning of the enzyme incubation; but with Ca^{2+} in the standard incubation mixture, the rate, though starting from a lower initial value, actually goes up with time upto about 7.5 min and then decreases but at a lower rate compared to the control. Therefore, from the kinetic point of view, Ca^{2+} induces a progressive activation of the prosta-glandin synthetase system at the initial stage which is exactly the opposite of auto-
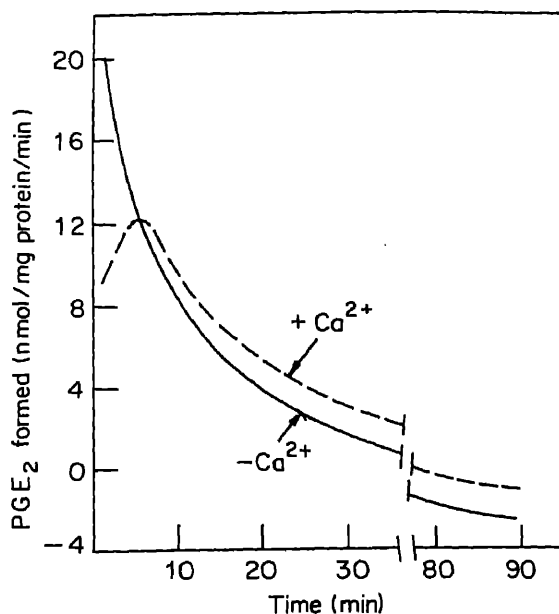
Figure 5. The effect of Ca^{2+} on the rate of prostaglandin E_2 biosynthesis with time. Tangents at various points on the corresponding curves in figure 4(a) drawn and the values of the slopes obtained were plotted against time.

inactivation normally observed under the same condition without Ca^{2+}; after the cross-over point at about 7 min, the Ca^{2+}-induced synthesis rate remains always at a higher level compared to the control, indicating some suppressing effect of Ca^{2+} on the autoinactivation of the prostaglandin synthetase system.

Discussion

The properties of the microsomal fraction of goat seminal vesicles have been studied in order to determine its suitability for enzymatic synthesis of prostaglandins. This investigation showed that the biochemical properties of the goat vesicular prostaglandin synthetase are very similar to those of the bull or sheep vesicular enzymes. This similarity of enzymatic properties suggests that bull, sheep or goat vesicular microsomes can be interchangeably used for experiments involving biosynthesis of prostaglandins.

During these studies, it became apparent that Ca^{2+} has a modulating effect on the PGE_2 synthetase activity. It has been previously shown that renal prostaglandin synthesis in the absence of Ca^{2+} is decreased significantly (Kalisker & Dyer, 1972). Very recently, Erman & Raz (1979) also reported stimulatory effects of Ca^{2+}, Sr^{2+} and Mn^{2+} on medullary synthesis of PGE_2 which they correlated with the stimulatory effects of the cations on arachidonic acid release from the tissue lipids. In addition they also observed a higher percentage of released arachidonate converted to PGE_2 when the cationic concentration was raised from 2 mM to 5 mM, which they thought was not due to any stimulatory effect of Ca^{2+} *per se* on medullary biosynthesis of PGE_2, but the effect of enhanced substrate concentration

through the activation of phospholipase A_2 available for the synthesis of the prostaglandin. However, our studies of prostaglandin biosynthesis *in vitro* did show a stimulatory effect of Ca^{2+} at about 4 mM concentration in the presence of excess substrate (figure 3) when the incubation period was long. It is perhaps significant to note that as in the case of medullary synthesis, *in vitro* prostaglandin formation catalyzed by goat vesicular microsomes is also stimulated most effectively by Ca^{2+} followed by Sr^{2+} at similar concentrations and not at all by Mg^{2+} But unlike the case of *in vivo* synthesis, no *in vitro* stimulation of the synthetase activity could be shown by Mn^{2+}. *In vitro* biosynthesis of PGE_2 is stimulated by Ca^{2+} and Sr^{2+} only upto about 50% (table 2) but the observed stimulation of the prostaglandin synthesis with tissue slices of medulla was much more pronounced (Erman & Raz, 1979). This difference in the extent of stimulation could be due to the enhancement by Ca^{2+} of (a) the availability of free arachidonic acid by activating phospholipase A_2 and (b) the activity of the synthetase system itself; results of the experiments of Kalisker & Dyer (1972) and Erman & Raz (1979) indirectly support such as possibility.

The biochemical basis of the Ca^{2+} effect on PGE_2 biosynthesis is quite unclear. Previous kinetic studies with the enzyme system showed that prostaglandin synthesis is controlled by the accelerating effect of low concentrations of an intermediate hydroperoxide (Cook & Lands, 1975, Hemler & Lands, 1980) and a self-catalyzed inactivating effect of cyclooxygenase itself on the reaction rate (Smith & Lands, 1972; Egan *et al.,* 1976). Whether the Ca^{2+} effect is due to a modulating activity on any of these well known positive and negative regulatory processes is now under investigation.

Acknowledgements

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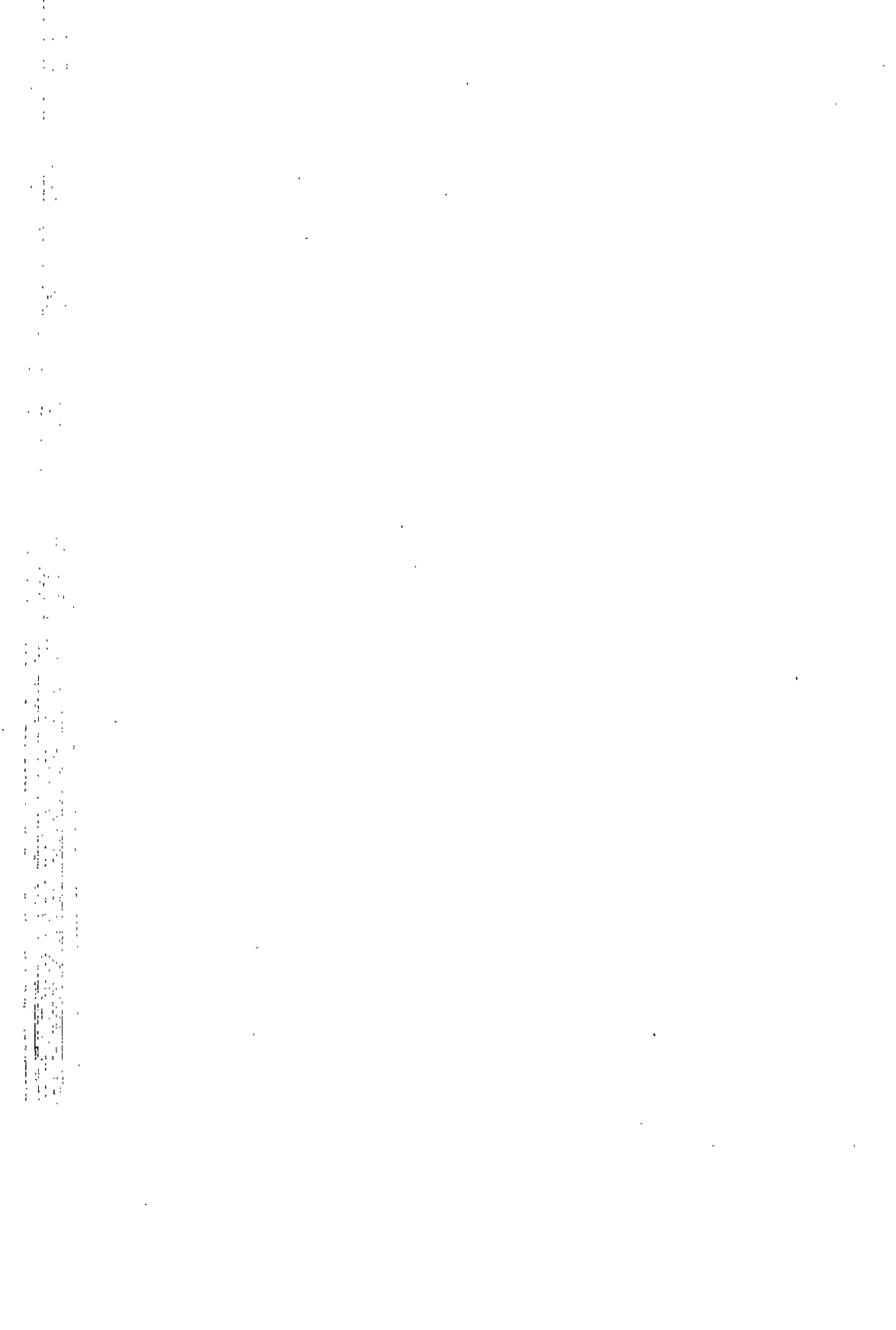
Changes in the content of glycogen and its metabolites during acute exposure of *A nabas testudineus* (Bloch) to furadan

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Abstract. Significant differences were observed in glycogen metabolism of *Anabas testudineus* exposed to an acute lethal (1.56 mg/litre) and a sublethal (0.56 mg/litre) concentration of furadan. At sublethal concentration, the muscle glycogen which was utilized during the early periods of exposure, was replenished in the later period of exposure and at 120 h, the muscle glycogen levels were higher than the control. At higher concentration, the liver glycogen levels showed an increase presumably at the expense of fuel reserves of the muscle.

Keywords. Acute toxicity; furadan; *Anabas testudineus;* glycogenesis; gluconeogenesis; glycogenolysis.

Introduction

Aquatic toxicologists use physiological dysfunction in fishes to assess the quality of water. This assessment to be meaningful, requires knowledge of the physiological aspects of fishes living in polluted waters (Gingerich and Weber, 1979). Though numerous studies have been carried out to understand the biochemical mechanisms underlying the chronic effects of pesticides on animals (Buhler and Benville, 1969; Mehrle *et al.,* 1971; Grant and Mehrle, 1973), no clear correlation between the acute changes in physiological processes and toxicity in fishes has emerged (Eisler and Edmund, 1966; Bakthavathsalam, 1980). Since the use of carbamate pesticides is extensive, their biochemical effects on fish are essential for establishing the criteria of water quality. With this objective, the acute effect of furadan on the liver and muscle glycogen content as well as a few parameters of glycogen metabolism in climbing perch, *Anabas testudineus* was examined.

Materials and methods

Specimens of climbing perch, *Anabas testudineus* (Bloch) of standard length (10-12 cm) and weight (8-20 g) collected from the fresh water ponds around Annamalainagar, Tamilnadu were maintained atleast for 15 days in large cement tanks (28

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±2°C) previously washed with potassium permanganate. The fish were fed every alternate day with boiled eggs and earthworms. A week before the commencement of experiments, a suitable number of healthy fish were transferred from the stock and maintained in the laboratory conditions (28 ±1°C) in small cement oisterns. These fish were fed every day and water in the cisterns was renewed daily. Feeding was stopped 2 days before the fish were used for experiments in order to reduce the amount of excreted products in the test tanks.

Technical grade furadan (2, 3-dihydro-2, 2-dimethyl-7-bensofuranyl methyl carbamate) manufactured by Rallis India Limited, Bangalore, was used. Stock solution of pesticide was prepared in acetone. Two concentrations, viz., 0.56 mg/litre (120 h LC_0) and 1.56 mg/litre (24 h LC_{100}) were chosen based on the acute toxicity test (Bakthavathsalam, 1980). Test solutions were prepared from the stock solution using filtered well-water with the following characteristics: temperature 28 ±1°C, pH 7.4-7.6, dissolved oxygen 7-10 ppm, salinity 0.2-0.6 ppm, alkalinity 240-260 mg/litre as $CaCO_3$ and hardness 360-380 mg/litre as $CaCO_3$.

About 15 fish were exposed to each selected concentration and at each exposure in 200 litre rectangular fibre glass tanks (100×50×40 cm). Acetone used in the test solution never exceeded 0.25 ml/litre of water at any concentration. Parallel controls were also maintained with a maximum aliquot of acetone (0.25 ml/litre) in the test chambers. The test solutions were renewed every 24 h.

Six surviving fish from each tank were sacrificed by decapitation after 1, 3, 6, 12, 24, 48, 72, 96 and 120 h in the case of control and 0.56 mg/litre of furadan treatment. In the case of 1.56 mg/litre of furadan treated lots, the fish were sacrificed at 1, 3 and 6 h of exposure. Muscle and liver were isolated in the cold and immediately frozen. Homogenates (5 and 10%) of liver and muscle respectively were prepared in 10% trichloroacetic acid and centrifuged at 1308 *g* for 15 min using Electrical Swing Head Covered Centrifuge and 1 ml of the supernatant was used for glycogen and lactic acid estimations. The blood samples (0.2 ml) were collected by caudal puncture using heparinized hypodermic syringe and were immediately deproteinized with 10 ml of 10% tungstic acid. The protein-free filtrates were used for glucose and lactic acid estimations.

Glycogen was estimated by the method of Carroll *et al.* (1956) using a Linear Readout Grating Spectrophotometer (Cecil model CE 373). Lactic acid was estimated, using Barker and Summerson's (1941) method. Blood glucose was determined by the method of Folin and Malmros microprocedure as modified by Murrel and Nace (1958). To minimise fluctuations due to diel rhythmicity (Datta Munshi and Patra, 1978) the experiments were carried out between 10.00 a.m. and 12.00 noon each day.

Statistical significance of the difference between the control and experimental values was calculated by Student's 't' test. Data were also subjected to one way analysis of variance (F test) as described by Steel and Torrie (1960).

Results

The mean content of muscle and liver glycogen (table 1), muscle and liver lactic acid (table 2) and blood lactic acid and blood glucose (table 3) for all the 9 periods

of control exposure were not significant (P>0.05) when subjected to analysis of variance (F test).

Table 1. Glycogen content in muscle and liver of *Anabas testudineus* treated with furadan.

Exposure (h)	Muscle glycogen[a]			Liver glycogen[a]		
	Control	0.56 mg/L	1.56 mg/L	Control	0.56 mg/L	1.56 mg/L
1	1.45±0.46	0.45±0.14	0.31±0.10	42.19±4.05	21.07±5.52	102.55±2.70
3	1.36±0.45[b]	1.15±0.11	0.18±0.04	41.17±2.12	6.32±1.17	86.00±17.66
6	1.29±0.44	1.19±0.25	0.35±0.19	40.57±1.42	105.22±4.05	101.50±4.91
12	1.61±0.42	0.68±0.34	—	40.37±0.77	67.04±4.36	—
24	1.41±0.43	0.50±0.13	—	40.02±0.90	1.80±0.25	1.80±0.25
24	1.41±0.43	0.50±0.13	—	40.02±0.90	1.80±0.25	—
48	1.23±0.44[c]	0.41±0.11	—	39.70±0.43	2.48±1.18	—
72	1.19±0.37	0.72±0.18	—	38.80±1.71	2.64±1.00	—
96	1.01±0.04	0.44±0.17	—	38.93±1.66	44.82±2.57	—
120	1.06±0.13	1.63±0.35	—	38.43±0.83	44.80±10.64[b]	—
F-value	1.96*	24.56**	3.17*	1.02*	366.81**	4.50**

[a] Values expressed as mg/g wet wt of tissue.
Mean ±S.D. of 6 individual observations;
[b] =P>0.05; b=P<0.05; Others P<0.01; * P_F>0.05; ** P_F<0.05. — Not determined.

Table 2. Lactic acid content of muscle and liver in *Anabas testudineus* treated with furadan.

Exposure (h)	Muscle lactic acid[a]			Liver lactic acid[a]		
	Control	0.56 mg/L	1.56 mg/L	Control	0.56 mg/L	1.56 mg/L
1	4.40±1.40	4.81±0.38[b]	2.30±0.43	2.08±0.61	2.73±0.46[b]	4.33±0.86
3	4.45±0.78	2.26±0.32	2.50±0.47	2.39±0.35	1.79±0.34[c]	2.38±0.37[b]
6	4.45±0.94	3.86±0.43[b]	4.19±0.30[b]	2.69±0.40	7.53±0.70	3.45±0.44[c]
12	4.50±0.50	5.42±0.82[b]	—	2.48±0.49	3.97±0.34	—
24	4.40±0.47	2.13±0.41	—	2.16±0.47	1.51±0.35[c]	—
48	4.45±0.51	2.89±0.42	—	2.52±0.55	2.13±0.55[b]	—
72	4.30±0.62	2.78±0.19	—	2.34±0.32	1.61±0.50[c]	—
96	4.10±1.00	2.83±0.30[c]	—	1.97±0.42	1.72±0.35[b]	—
120	3.90±1.04	3.61±0.22[b]	—	2.20±0.35	1.93±0.49[b]	—
F-value	1.73*	41.18**	40.04**	1.34*	104.52**	16.01**

[a] Values expressed as mg/g wet wt. of tissue.
Mean ±S.D. of 6 individual observations;

[b] =P 0.05; [c]=P<0.05; Others P<0.01; * P_F>0.05; ** P_F<0.05. — Not determined.

Table 3. Lactic acid and glucose contents of blood in *Anabas testudineus* treated with furadan.

Exposure (h)	Lactic acid[a]			Glucose[a]		
	Control	0.56 mg/L	1.56 mg/L	Control	0.56 mg/L	1.56 mg/L
1	1.00±0.39	2.19±0.60[c]	2.58±0.84	6.25±0.71	11.47±0.95	14.42±0.38
3	1.01±0.29	2.13±0.57	3.77±0.84	6.06±0.65	11.46±0.53	8.50±0.72
6	1.19±0.46*	2.65±1.12	7.02±1.42	6.25±0.59	21.46±0.43	20.83±1.16
12	1.00±0.35	5.94±1.18	—	6.33±0.81	12.68±0.99	—
24	1.05±0.31	1.13±0.53[b]	—	6.38±0.89	6.25±1.06[b]	—
48	1.11+0.17	2.17±0.75	—	5.88±1.31	12.64±0.64	—
72	1.11±0.26	3.65±0.71[c]	—	5.90±1.03	8.50±0.47	—
96	0.93±0.21	3.96±0.69	—	6.14±0.91	16.92±0.80	—
120	0.95±0.13	1.73±0.86[b]	—	4.51±1.25	15.00±0.85	—
F-value	1.63*	19.51**	27.71**	1.08*	198.67**	341.99**

[a] mg/ml of blood

Mean ±S.D. of 6 individual observations;

[b] =P 0.05; [c] =P>0.05; Other P<0.01; * P_F>0.05; ** P_F<0.05. — Not determined.

In 0.56 mg/litre furadan exposure, a significant decrease (P<0.01) at 1 h (followed by a recovery during 3 and 6 h was observed in the muscle glycogen content of fish (table 1). From 12 h onwards, marked decrease was noted. But a significant (P<0.01) increase was noted in the muscle glycogen at 120 h of exposure.

After 1 h exposure, the liver glycogen content showed 50% decrease, which was further reduced to 85% at 3 h. A sudden increase (P<0.01) was noticed in the glycogen level at 6 h which followed an acute drop until 72 h of exposure. The glycogen content attained normal level during 96 h and it was maintained at 120 h also (table 1).

Blood glucose showed a marked increase at 6 h and 120 h (table 3) which coincided with periods of highest glycogen content in liver and muscle. Lactic acid content of muscle (table 2) showed a greater depletion at later periods of exposure from 24 to 120 h when the liver glycogen content was at low levels. Liver lactic acid (table 2) content followed the changes in the liver glycogen content in the initial periods of exposure. For example during 1 h, the increase was 31.3% but in 3 h a decrease of 25.1% was noticed, when liver glycogen also showed a remarkable decrease. At 6 and 12 h of exposure, lactic acid content of liver showed an increase in its levels at all periods of exposure but the increase observed at 12, 96, 72 and 16 h are noteworthy (table 3).

Anabas testudineus exposed to 1.56 mg/litre furadan showed a marked decrease in muscle glycogen and lactic acid contents at all periods. Liver glycogen and lactic acid and blood glucose and lactic acid, on the other hand, showed an upward shift in their levels at all the three periods of exposure (tables 1, 2 and 3).

Further, the changes observed in the mean content of various metabolites at both the concentrations were statistically significant ($P < 0.05$) except muscle glycogen contents of fish exposed to 1.56 mg/litre furadan, when analysis of variance was applied on them.

Discussion

Glycogen content of muscle and liver decreased to a greater extent viz., 69.0 and 50.1% respectively (table 1) at 1 h with 0.56 mg/litre of furadan exposure compared to similar exposure to disyton, (O, O-diethyl S-[2-(ethylthio)-ethyl] phosphoro dithioate) (Bakthavathsalam, 1980).   Concomitant with this active glycolytic metabolism, the muscle, liver, and blood lactic acid levels were high in furadan-treated fish.   Liver glycogen levels were further decreased at 3 h whereas muscle glycogen levels were slightly improved.   The fall in muscle and liver lactic acid levels suggested that glycogenesis should have prevented the otherwise signi-ficant reduction of liver glycogen.   But at 6 h, the presumable depletion of liver glycogen was averted, the glycogen levels being considerably improved showing an increase of 158.4% over control.   Blood glucose levels were highest at 6 h emphasizing an adverse effect on glycogen metabolism.   Liver lactic acid levels were also high (table 2) and since muscle and liver lactic acid levels at 3 h could not quantitatively account for this apparent rise, it could be safely assumed that either lactic acid formed in other tissues was transported to liver or it was a product of liver metabolism itself or a combination of both was responsible for high lactic acid levels in the liver at 6 h.

Both muscle and liver glycogen levels were low at 12 h with an apparent increase in lactic acid content of blood and muscle.   Blood glucose and liver lactic acid also decreased to 100.3 and 60.1% respectively.   All these changes evidently suggest extensive utilisation of glycogen leading to a serious reduction in the glycogen content of liver and muscle, lactic acid content of muscle, liver and blood, and blood glucose at 24 h when compared to their levels in 12 h of exposure.   The insignificant change ($P > 0.05$) in the muscle and liver glycogen levels at 48 h over 24 h values may not mean that the glycogen metabolism may have reached a steady state.   The rate of utilization probably equalled the rate of supplementation. Increase in blood glucose and blood lactic acid and a fall in muscle and liver lactic acid all contribute to an active flux of metabolites.   In the subsequent periods of exposure, the glycogen stores were slowly repleted, the improvement first starting in the muscle as reflected by the change in the mean glycogen content from 0.41 at 48 h to 0.72 mg/g at 72 h.

But at 72 h, though muscle lactic acid did not change much, high blood lactic acid content indicated transport of large amounts of lactic acid formed in muscle and/or other tissues to liver.   This event probably led to a decrease in muscle glycogen and rise in liver glycogen at 96 h.   Blood lactic acid continued to be high at 96 h which perhaps helped to maintain liver glycogen and increase in muscle glycogen at 120 h in the face of sustained high transport of glucose at 96 h. Increase in blood glucose at 96 h and 120 h probably was the reason for the upward shift in the muscle glycogen levels observed at 120 h.   The elevation observed in glycogen content of fish after 120 h of exposure may be an indication for successful

adaptation to furadan toxicity or may be a response to an abnormal increase in succinate dehydrogenase activity levels observed in various tissues of fish at this period (Bakthavathsalam, 1980).

In contrast to 0.56 mg/litre furadan exposure, 1.56 mg/litre exposure was characterized by significant changes in muscle and liver glycogen levels in singular but opposite directions. The decrease in muscle glycogen might be due to the rate of utilization gaining upperhand over the rate of supplementation. If the blood lactic acid and blood glucose were taken to reflect the former and the latter, respectively, the difference in the per cent increases observed in the two, makes this point clear (table 3). The increase observed in liver glycogen and blood glucose may be due to the inhibition of glycogenolysis or due to the initiation of gluconeogenesis and/or glycogenesis. These results are in agreement with the findings of Grant and Mehrle (1973) that glycogenolysis was inhibited by the high endrin concentration, or that gluconeogenesis and glycogenesis were greatly increased, resulting in high liver glycogen content. Further high blood glucose and liver glycogen in 1.56 mg/litre treated group suggests an impairment in the carbohydrate metabolism.

Acknowledgements

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Bacitracin A: Structure-activity relationship

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Abstract. The single imidazole nucleus of L-histidine residue in bacitracin-A seems to be important for the anti-bacterial activity of the molecule, since iodination, carboxymethylation and coupling of diazobenzene sulphonic acid to the histidine residue in the antibiotic caused 90-94% loss of antibacterial activity of the antibiotic. In contrast, the bacitracin sulphone and sulphoxide derivatives are as active as the parent antibiotic.

Keywords. Bacitracin-A; chemical modification; iodination; carboxymethylation; diazotization.

Introduction

Previous investigations from this laboratory (Rambhav, 1981) on bacitracin-A have revealed that (i) the single chain amino group of D-ornithine residue is necessary for the anti bacterial activity on the molecule and (ii) the α- and γ-carboxyl groups of D-asparagine and D-glutamic acid residues of the molecule are not required for the antibacterial activity of the antibiotic. The present study deals mainly with the effect of iodination, carboxymethylation and coupling of diazobenzene sulphonic acid moiety to the imidazole group of the single L-histidine residue of the antibiotic on the antibacterial activity of the molecule. The oxidation of the sulphur atom in the thizoline ring of bacitracin-A to the level of sulphoxide and sulphone on the antibacterial activity is also discussed.

Materials and methods

Bacitracin (58.4 units/mg, Calbiochem, Los Angeles, California, USA) was a gift from Prof. L. K. Ramachandran. All reagents and solvents obtained from commercial sources were purified as and when necessary. The diazobenzene sulphonic acid was prepared from sulphonilic acid (Pauly, 1904).

All colorimetric measurements were carried out with a Systronics double cell colorimeter, type 102 or with a ELICO photoelectric colorimeter, CL-20. Spectrophotometric measurements were done with a Toshniwal Spectrophotometer. The antibiotic content in bacitracin derivatives was based on weight, and where necessary checked by Pauly's reaction for imidazole group (Pauly, 1904). Diazo-

benzene sulphonic acid groups were estimated both by the determination of the total acid groups (Fraenkel-Conrat and Cooper, 1944) of the original and modified antibiotic; and by spectral analysis (Tabachnick and Sobotka, 1960).

Iodine content in the iodobacitracin was estimated by the alkali fusion method (Kendall, 1914). The extent of carboxymethylation of bacitracin was ascertained by titrating a known amount of the derivative against a standard alkali using phenol-phthalein indicator. Antibacterial activity of modified bacitracins was deter-mined using two Gram-positive organisms, *Streptococcus faecalis* (ATCC 8043) and *Staphylococcus aureus* (NCIM 2079) as before (Rambhav, 1981).

Results

Preparations of modified bacitracins

Iodobacitracin: Bacitracin (30 mg) was dissolved in water (1.0 ml) and cooled to 0-4°C in an ice bath. The pH of the solution was adjusted to 5.5 with the addition of a few drops of acetic acid. The solution was treated with the iodine solution (0.1 ml, 0.1M I$_2$ in 0.5M KI) at 5 min intervals and stirred well in cold. A total of 1.0 ml of the reagent was added, maintaining the pH around 5.5 and left in the cold over-night. The contents were diluted to about 30 ml with cold acetone, mixed well and left in cold for about 2 h. The product was isolated by filtration and washed with a small quantity of cold acetone and finally dried under vacuum over sodium hydroxide pellets. The yield was 16 mg and the iodine content was 0.82 µmol/mol of antibiotic.

Carboxymethyl bacitracin: An aqueous solution of bacitracin (30 mg in 1.0 ml, adjusted to pH 5.5 with acetic acid) was treated with 0.1M aqueous solution of iodoacetate. A total of 2.0 ml of the reagent was added during the period of 1 h in the cold, maintaining the pH around 5.5. The reaction mixture was left in the cold overnight and later the product was precipitated by the addition of nearly 30 ml of cold acetone. The product was isolated by filtration. The yield was 19.8 mg and the extent of carboxymethylation was 78%.

Coupling of bacitracin with diazobenzene sulphonic acid

An aqueous solution of bacitracin (30 mg in 1.0 ml) was cooled to 0°C in an ice bath and the pH of the solution was lowered to 6.5 by the addition of a drop of dilute acetic acid. The reaction was initiated by the addition of the freshly prepared diazobenzene sulphonic acid (0.5 ml, 0.05 mmol per ml). A total volume of 4.0 ml of reagent was added during a period of 1 h in the cold with continuous stirring and maintaining the pH around 6.5 Later the reaction was allowed to proceed in the cold for about 18 h. The reaction mixture was then diluted with cold acetone to about 40 ml to precipitate the product. The product was isolated by filtration. The yield was 22 mg.

Bacitracin sulphone

To 30 mg of bacitracin in 1.0 ml of water, was added a few drops of dilute acetic acid to lower the pH to 6.0-6.2 and cooled to 0-5°C. A solution of 16 mg of

potassium permanganate in 2.0 ml of water and 0.1 ml phosphoric acid was added to the bacitracin solution during a period of 30 min in cold maintaining the pH around 6.0. After 30 min, excess of potassium permanganate was destroyed by the addition of few mg of sodium bisulphate. The reaction mixture was diluted to about 30 ml with the addition of cold acetone to precipitate the product. The product was isolated by centrifugation and an yield of 39.8 mg was obtained.

Bacitracin sulphoxide

An aqueous solution of bacitracin (30 mg in 1.0 ml) was cooled to 0°C and the pH of the solution was lowered to 6.0 by adding a few drops of dilute acetic acid. An aqueous solution of sodium metaperiodate (12 mg in 1.0 ml) was added to the bacitracin solution and the reaction mixture was stirred well in cold for about 2 h maintaining the pH around 6.0. Later a few drops of phosphoric acid was added to lower the pH to 4.0 and left in the cold for about 2 h. The reaction mixture was then diluted to about 30 ml by the addition of cold acetone, and again left in cold for about 1 h to precipitate the product. The product was isolated by filtration. The derivatives were purified by gel filtration on a column of Sephadex G-10 (1.1×14 cm), equilibrated with 5% acetic acid and yield was 48 mg. The presence of bacitracin sulphone or suloxide derivative was tested in the column fractions by the amino group estimation by ninhydrin method (Rosen, 1957).

Properties of modified bacitracin

The absorption spectrum of diazobenzene sulphonic acid coupled bacitracin in the visible region is shown in figure 1. The derivative showed a molar extinction
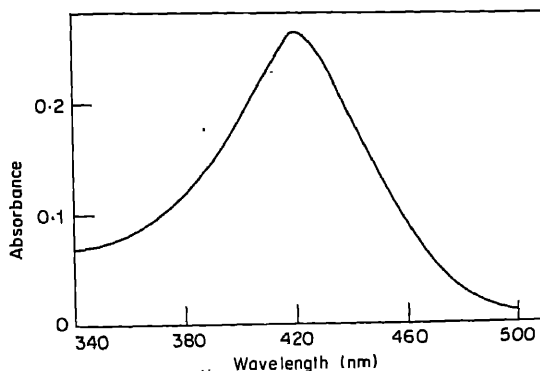
Figure 1. Absorption spectrum of diazobenzene sulphonic acid coupled bacitracin.

coefficient of 26,500 at 420 nm in 0.1N sodium hydroxide solution. Data reported for monoazo histidine is 23,400 at 423 nm in 0.1N sodium hydroxide solution (Tabachnick and Sabokta, 1960). The analysis showed a content of 1.13 µmol of diazobenzene sulphonic acid groups per µmol of the antibiotic. Bacitracin sulphone and sulphoxide derivatives were chromatographically homogeneous and their mobilities (R_f values) were different from that of the native antibiotic (Table 1).

Chromatographic homogeneity of bacitracin derivatives

All modified bacitracins were chromatographed (descending chromatography in n-butanol: acetic acid: water (3:1:1), and gave single spots with ninhydrin reagent spray. The R_f values of the modified bacitracins are shown in table 1.

Table 1.   Mobilities (R_f values) of Bacitracin and its derivatives

Sample	Solvent (*n*-butanol:acetic acid:water; 3:1:1)
Bacitracin	0.55
Iodo-bacitracin	0.48
Carboxymethyl-bacitracin	0.37
Diazobenzene sulphonic acid coupled bacitracin	0.60
Bacitracin-sulphone	0.16
Bacitracin-sulphoxide	0.25

Antibacterial activity

The results on the antibacterial activity of bacitracin and its derivatives are shown in table 2.   The 50% inhibitory concentrations ($I_{50\%}$) for bacitracin in the assay, using *S. faecalis* and *S. aureus,* were 0.4 and 2.0 µg/ml, respectively.   Iodination, carboxymethylation and coupling of diazobenzene sulphonic acid moiety to the histidine residue resulted in 90-94% loss of antibacterial activity.   Bacitracin sulphone and sulphoxide derivatives, in contrast, were 84-97% as active as bacitracin.

Table 2.   Antibacterial Activity of Bacitracin and Its Derivatives

Sample	*S. faecalis*		*S. aureus*	
	$I_{50\%}$ (µg/ml)	Activity (%)	$I_{50\%}$ (µg/ml)	Activity (%)
Bacitracin	0.4	100	2.0	100
Iodo-bacitracin	10.0	4	25.0	8
Caboxymethyl-bacitracin	8.5	5	28.0	7
Diazobenzene sulphonic acid coupled bacitracin	10.5	4	20.0	10
Bacitracin-sulphone	0.4	93	2.2	91
Bacitracin-sulphoxide	0.4	97	2.4	84

The $I_{50\%}$ values represent the average of three experiments.

Discussion

Bacitracins are closely related mixtures of bactericidal polypeptide antibiotics obtained from *Bacillus subtilis*. Bacitracins are active against numerous Gram-positive bacteria. The type of modifications studied in the present investigation are iodination, carboxymethylation and coupling of diazobenzene sulphonic acid residue to the single histidine residue in the antibiotic. The sulphur atom in the thiazoline ring of the molecule was also oxidized and the effects on its antibacterial activity are studied.

Bacitracin is stable only in the pH range 4.0-6.0. Hence all chemical modifications performed on the molecule have been carried out between pH 4.0-6.0, to ensure that stability of the rest of the molecule is unaltered. The property of insolubility of bacitracin in acetone was used to precipitate the derivatives from the reaction mixture. The purity of the derivatives was ascertained by paper chromatography. The degree of modification has been provided based on direct and/or indirect methods. A study of the antibacterial activity has revealed the essentiality of the intact basic imidazole nucleus of histidine residue, since its iodination, carboxymethylation and coupling of diazobenzene sulphonic acid residue results in 90-94% loss of activity. On the otherhand, the sulphur atom in the thiazoline ring of the molecule appears to be unimportant, atleast as regards antibacterial activity, since bacitracin sulphone and sulphoxide derivatives are as active as the unmodified antibiotic.

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Purification, physicochemical and regulatory properties of serine hydroxymethyltransferase from sheep liver

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Abstract. Serine hydroxymethyltransferase (EC 2.1.2.1) was purified from the cytosolic fraction of sheep liver by ammonium sulphate fractionation, CM-Sephadex chromatography, gel filtration using Ultrogel ACA 34 and Blue Sepharose affinity chromatography. The homogeneity of the enzyme was rigorously established by polyacrylamide gel and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, isoelectrofocusing, ultracentrifugation, immunodiffusion and immunoelectrophoresis. The enzyme was a homotetramer with a molecular weight of 210,000 ±5000. The enzyme showed homotropic cooperative interactions with tetrahydrofolate ($n_H = 2.8$) and a hyperbolic saturation pattern with L-serine. At the lowest concentration of tetrahydrofolate used (0.2 mM), only 5% of the added folate was oxidized during preincubation and assay. The n_H value was independent of the time of preincubation. Preincubation of the enzyme with serine resulted in a partial loss of the cooperative interactions ($n_H = 1.6$) with tetrahydrofolate. The enzyme was regulated allosterically by interaction with nicotinamide nucleotides; NADH was a positive effector while NAD$^+$ was a negative allosteric effector. The subunit interactions were retained even at the temperature optimum of 60°C unlike in the case of the monkey liver enzyme, where these interactions were absent at higher temperatures. D-Cycloserine, a structural analogue of serine caused a sigmoid pattern of inhibition, in contrast with the observations on the monkey liver enzyme. Cibacron blue F3GA completely inhibited the enzyme and this inhibition could be reversed by tetrahydrofolate. Unlike in the monkey liver enzyme, NAD$^+$ and NADH gave considerable protection against this inhibition. The sheep liver enzyme differs significantly in its kinetic and regulatory properties from the serine hydroxymethyltransferases isolated from other sources.

Keywords. Serine hydroxymethyltransferase; allostery; tetrahydrofolate.

Introduction

Although serine hydroxymethyltransferase (5,10-methylene-tetrahydrofolate-glycine hydroxymethyltransferase, EC 2.1.2.1) from bacterial (Mansouri *et al.,* 1972) and animal (Schirch, 1971; Nakano *et al.,* 1968; Fujioka, 1969; Jones and Priest, 1976; Ulevitch and Kallen, 1977a) sources has been studied extensively, it became known only recently that the enzyme from monkey liver (Ramesh and Appaji Rao, 1980a), isolated by a milder procedure, was a regulatory enzyme under the allosteric modulation of nicotinamide nucleotides (Ramesh and Appaji Rao, 1978). It was also observed that the partially purified enzyme from pig kidney

showed homotropic cooperative interactions with tetrahydrofolate and that this property was absent in the enzyme in cancerous tissues (Harish Kumar *et al.*, 1976). Since the enzyme occupies a key metabolic position in the supply of one-carbon fragments to the different coenzyme forms of folic acid, it could be predicted that this enzyme is regulatory in most organisms. Studies on the monkey liver enzyme also showed that Cibacron blue F3GA, which is used as an immobilized ligand in the purification of a large number of proteins containing the 'nucleotide fold', was a linear competitive inhibitor when tetrahydrofolate was the varied substrate, suggesting interaction at the tetrahydrofolate binding domain (Ramesh and Appaji Rao, 1980b). A recent study with dihydrofolate reductases isolated from chicken liver and *Lactobacillus casei* showed several interesting differences between the enzymes from these two sources in their mode of interaction with the dye (Subramanian and Kaufman, 1980). In view of the uniqueness of the initial discoveries with the monkey liver serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1978, 1980a, b; Ramesh *et al.*, 1981a, b), it was of interest to isolate the enzyme from another mammalian source, such as sheep liver and examine its regulatory properties and its interaction with the dye Cibacron blue.

Materials and methods

Chemicals

All the chemicals used were of Analytical grade or purchased from Sigma Chemical Co., St. Louis, Missouri, USA, except CM-Sephadex C-50 and Blue Sepharose CL6B which were from Pharmacia Fine Chemicals, Uppsala, Sweden; Freund's complete adjuvant and agar from Difco Laboratories, Detroit, Michigan, USA; tetrahydrofolate was prepared by the method of Hatefi *et al.* (1959) and was also obtained as a kind gift from Dr. J. H. Mangum, Brigham Young University, Provo, Utah, USA; dichloromethotrexate and aminopterine were a kind gift from Dr. R. Silber, New York University Medical Centre, New York, USA; Ultrogel AcA-34 was a product of Industrie Biologique Franceise gifted by Mr. Henrik Perlmutter, LKB Products AB, Stockholm, Sweden; Cibacron blue F3GA was a kind gift from Ciba-Geigy, Basel, Switzerland and DL-[-^{14}C] serine (48.5 mCi/mmol) was from New England Nuclear, Boston, Massachusetts, USA.

Animals

Liver from healthy sheep was obtained from a local abattoir immediately after slaughter and chilled crushed ice.

Assay of serine hydroxymethyltransferase

The enzyme was assayed according to the method of Taylor and Weissbach (1965) with some modification. The usual components of the assay mixture (0.1 ml) were, 0.4 M potassium phosphate buffer, pH 7.4; 1 mM 2-mercaptoethanol; 1 mM EDTA; 0.1 mM pyridoxal 5'-phosphate; DL-[3-^{14}C] serine was diluted with 3.6 mM L-serine and 1.2×10^5 cpm were added; 1.8 mM tetrahydrofolate; 1.8 mM dithiothreitol and an appropriate amount of the enzyme. After preincubation for 5 min at 37°C, the reaction was initiated by the addition of serine. After incubation for 15 min at 37°C the reaction was stopped with 0.1 ml of freshly prepared dimedon

(0.4 M in 50% (v/v) ethanol), the mixture heated for 5 min at 100°C and the [^{14}C]-formaldehyde-dimedon adduct extracted into toluene and the radioactivity measured in a Beckman LS-100C liquid scintillation spectrometer. One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 μmol of formaldehyde/min. Specific activity was expressed as units/mg protein.

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Tetrahydrofolate concentration was determined by spectral (Hatefi *et al.*, 1959); enzymatic (Ramesh *et al.*, 1978) and chemical methods. (Huennekens *et al.*, 1963).

Purification of sheep liver cytosolic serine hydroxymethyltransferase

The potassium phosphate buffer used in this study always contained 1 mM EDTA; 0.1 mM pyridoxal 5'-phosphate; and 1 mM 2-mercaptoethanol. Preliminary studies (data not given) showed that the enzyme was located in the cytosolic fraction of the sheep liver homogenate. The entire process of purification was carried out at 0-4°C.

Ammonium sulphate fractionation

Liver tissue (150 g) was cut into small pieces and homogenized in 400 ml of 0.05 M phosphate buffer in an industrial Waring Blendor. The homogenate was centrifuged at 27,000 *g* for 20 min. The supernatant crude extract was fractionated with ammonium sulphate at 25 to 50% saturation and the precipitate obtained was suspended in a minimal amount of 0.05 M phosphate buffer and dialyzed against the same buffer (about 400 volumes).

CM-Sephadex C-50 chromatography

The dialyzed ammonium sulphate fraction was applied onto a CM-Sephadex C-50 column (2.5×36 cm), equilibrated with 0.05 M phosphate buffer. After washing the column with buffer until the absorbance of the eluate decreased to less than 0.05; the enzyme, bound as a yellow zone at the top of the column was eluted with a linear gradient (0.05 M to 0.5 M) of potassium phosphate buffer at a flow rate of about 20 ml/h. Fractions (2 ml) of the eluate, having considerable activity (>0.7 units/mg) were pooled and concentrated to about 1 ml by precipitation with ammonium sulphate (60% saturation) followed by dialysis against 0.05 M phosphate buffer.

Molecular exclusion chromatography on Ultrogel AcA-34

The concentrated enzyme solution (approx. 1 ml) was passed through an ultrogel AcA-34 column (110×1.5 cm) equilibrated with 0.2 M phosphate buffer, at a flow rate of about 10 ml/h. The fractions (2 ml) having more than 50% of the activity of the peak fraction were pooled.

Affinity chromatography on Blue-Sepharose CL-6B

The enzyme was loaded on a Blue-Sepharose CL-6B column (1×20 cm), equilibrated with 0.02 M phosphate buffer. After washing the column with about 75 ml of the same buffer, the enzyme was eluted with buffer containing 1 M KCl at a flow rate of about 20 ml/h. The fractions (2 ml) containing the enzymatic activity

were pooled and dialyzed against 0.05 M phosphate buffer. This preparation at a concentration of 1-1.5 mg/ml was divided into small volumes and stored at −40°C. Freshly thawed enzyme was used in each experiment. The results of a typical purification are summarized in table 1.

Table 1. Purification of sheep liver cytosolic serine hydroxymethyltransferase.

Step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Fold purification	Per cent recovery
Crude	21609	986	0.046	1	100
Ammonium sulphate fractionation	12294	777	0.063	1.4	79
CM-Sephadex chromatography	128	181	1.42	31	19
Ultrogel filtration	34	196	5.75	125	20
Blue Sepharose affinity chromatography	27	162	6.03	131	16

Weight of liver tissue=150 g.

* μmol HCHO formed/min at 37°C and at pH 7.4.

Electrophoresis

Analytical polyacrylamide gel disc electrophoresis was done at 4°C in both 5% and 7.5% gels in 0.5 M Tris-0.39 M glycine buffer, pH 8.6, at a current of 4 mA per gel (Davis, 1964). Protein bands in the gels were stained with Coomassie Brilliant Blue G (0.02% in 3.5% perchloric acid).

Sodium dodecylsulphate polyacrylamide gel electrophoresis was done in 7.5 and 12.5% gels according to the method of Weber and Osborn (1969), both in the presence and in the absence of 2-mercaptoethanol. The marker proteins used to determine subunit molecular weight were, undissociated immunoglobulin, IgG (147,600), conalbumin (86,000), the H chain of IgG (49,000), ovalbumin (43,000), the L chain of IgG (24,000) and a-chymotrypsin (24,000). Isoelectric focusing in 5% gel was by the method of O'Farrel (1975).

Determination of molecular weight by gel filtration

The molecular weight of the native enzyme was determined by the gel filtration method of Andrews (1965) using a Sephadex G-200 column (1.5×50 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.4 and operated at a flow rate of 15 ml/h. The enzyme was monitored by measuring the activity. The standard protein markers used were bovine serum albumin (68,000), ovalbumin (43,000, lysozyme (14,400), chymotrypsin (24,000), myoglobin (17,000) and bovine type I thyroglobulin (660,000).

Ultracentrifugation

The sedimentation velocity of the purified enzyme (8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4, was determined in a Spinco Model E Analytical Ultracentrifuge, using a single sector cell and Schlieren optics. The operating speed was 39,460 rpm and the temperature, 20°C. The $S_{20,w}$ was calculated (Schachman, 1957).

Immunological techniques

Antiserum to the purified enzyme was raised in the rabbit. The enzyme (750 µg) in Freund's complete adjuvant was injected intradermally into a rabbit. Four weeks later, about 350 µg of the protein in Freund's incomplete adjuvant was injected intradermally. After a further four weeks, a booster dose of 150 µg of the enzyme was administered in saline, intravenously. The animal was bled from the marginal ear vein on the seventh and ninth days after the last injection. The serum was separated from the blood clot and used as the antiserum.

Ouchterlony double diffusion analysis was done (Ouchterlony, 1958) in an agar plate. Antiserum (100 µl) was placed in the central well and different concentrations of the enzyme (5 µg to 30 µg/100 µl) were placed in the surrounding wells.

Immunoelectrophoresis was done in 1.25% agar with 0.05 M potassium phosphate buffer, pH 7.4 in the electrode chambers (Williams and Graber, 1955).

Spectroscopic methods

The absorption spectrum of the enzyme (1.28 mg/ml) both in the ultraviolet (uv) and in the visible ranges were obtained with a Beckman Model 26 spectrophotometer. Difference spectra were taken against 0.05 M potassium phosphate buffer, pH 7.4 containing 0.1 mM pyridoxal 5'-phosphate; 1 mM 2-mercaptoethanol and 1 mM EDTA.

The fluorescence spectra of the enzyme (50 µg/ml) were obtained in a Hitachi Perkin-Elmer spectrophotometer after dialyzing out pyridoxal 5'-phosphate against 0.05 M potassium phosphate buffer, pH 7.4 containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The same buffer was used as blank.

The circular dichroic (c.d.) spectrum of the native enzyme was obtained in a Jasco 20A spectropolarimeter after dialyzing out the pyridoxal 5'-phosphate as before. The protein concentration and path length were 0.885 mg/ml and 10 mm for the visible and near uv regions and 0.3 mg/ml and 1 mm for the far uv region, respectively. The helical content was calculated according to Greenfield and Fasman (1969).

Results

Purification of the enzymes

Table 1 summarizes the results of a typical purification procedure for the isolation of the sheep liver serine hydroxymethyltransferase. The enzyme fraction obtained from the ultrogel column was only slightly contaminated and this contaminating

protein was eliminated by using the Blue Sepharose affinity matrix (figure 1a). The enzyme elutes from the Blue Sepharose column as a single symmetrical protein peak corresponding to the activity peak (data not given). With a starting material of 150 g of tissue, the method described above yields 22-28 mg of the enzyme of specific activity 6 ± 0.2 units/mg protein, purified about 130-fold, with a recovery of 15-20%. Stored at −40°C, the enzyme showed no appreciable loss of activity for over two months.
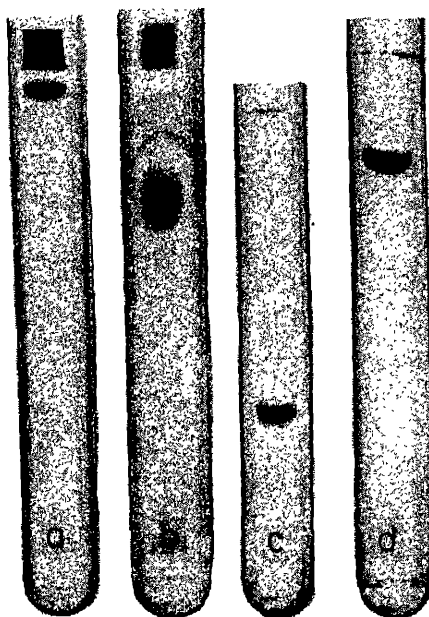
Figure 1. Analytical polyacrylamide and SDS-polyacrylamide gel electrophoresis.

Figure 1a and 1b represent electrophoresis of the purified enzyme (50 µg) on 7.5% and 5% polyacrylamide gels, respectively. Electrophoresis was done at 4°C in 0.5 M Tris−0.39 M glycine buffer, pH 8.6, at a current of 4 mA/gel. The protein bands were stained with Coomassie Brilliant Blue-G. Figure 1c and 1d represent sodium dodecyl sylphate-polyacrylamide gel electrophoresis of the purified enzyme in 7% and 12.5% gels respectively. The enzyme (200 ug) was treated with sodium dodecyl sulphate (2%) and heated for 3 min at 100°C. An aliquot corresponding to 30 µg protein was subjected to electrophoresis at a current of 4 mA/tube. The gels were stained with Coomassie Brilliant Blue R and destained with 10% acetic acid.

Criteria of homogenity

Upon electrophoresis in 7.5% polyacrylamide gel, the enzyme preparation (30-50 µg) gave a single protein band of very low mobility (figure 1a); on a 5% gel the protein migrated more freely but gave a diffused band (figure 1b). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the enzyme also resulted in a single protein band in both 7% and 12.5% gels (figure 1c and 1d). Isoelectric focusing of the enzyme preparation resulted in a single protein band corresponding to a pI of 4.2.

Upon ultracentrifugation, the enzyme sedimented as a single sharp symmetrical peak (figure 2a). The sedimentation coefficient value ($S_{20,w}$) was calculated to be 6.8.

A single precipitin line was obtained in the Ouchterlony immunodiffusion test at all the concentrations (5-30 µg/100 µl) of the enzyme. The ends of the precipitin lines joined to form an uniform hexagon (figure 2b). Immunoelectrophoresis of the enzyme also resulted in a single precipitin line (data not given).
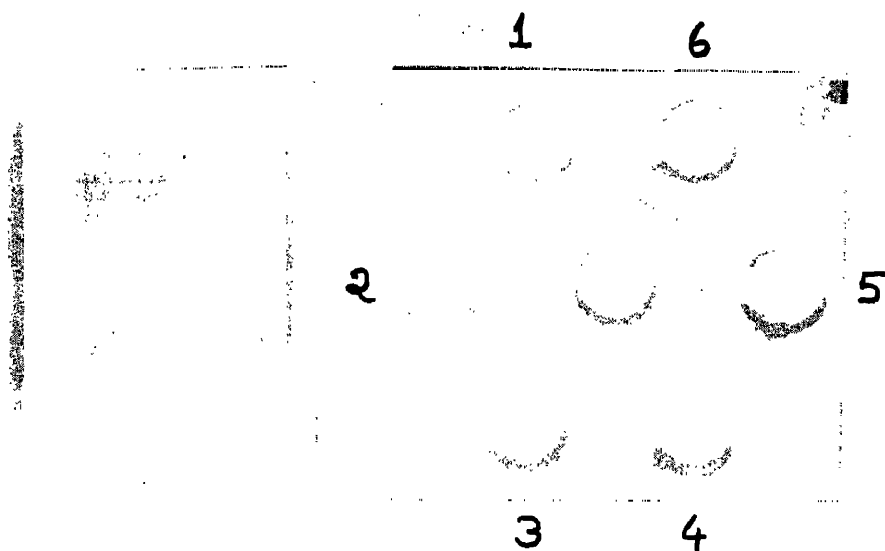
Figure 2a. Sedimentation pattern of sheep liver serine hydroxymethyltransferase.

The ultracentrifugation analysis was done with a purified preparation of the enzyme (8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4, in a Spinco Model E Analytical Ultra-centrifuge provided with Schleiren optics. The operating speed was 39,460 rpm and the temperature, 20°C. The photograph was taken 75 min after the attainment of this speed.

Figure 2b. Ouchterlony immunodiffusion analysis of sheep liver serine hydroxymethyl-transferase.

Antiserum (100 µl) was placed in the central well and different concentrations of the purified enzyme (5, 10, 15, 20, 25 and 30 µg /100 µl) were placed in the surrounding wells (1—6).

Determination of molecular weight and subunit composition of the enzyme

The molecular weight of the native enzyme estimated by gel filtration using a calibrated Sephadex G-200 column was found to be 210,000 ±5000 (figure 3).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, both in the presence and in the absence of 2-mercaptoethanol, gave rise to a single protein band corresponding to a molecular weight of 55,000 ±2500 (figure 3).
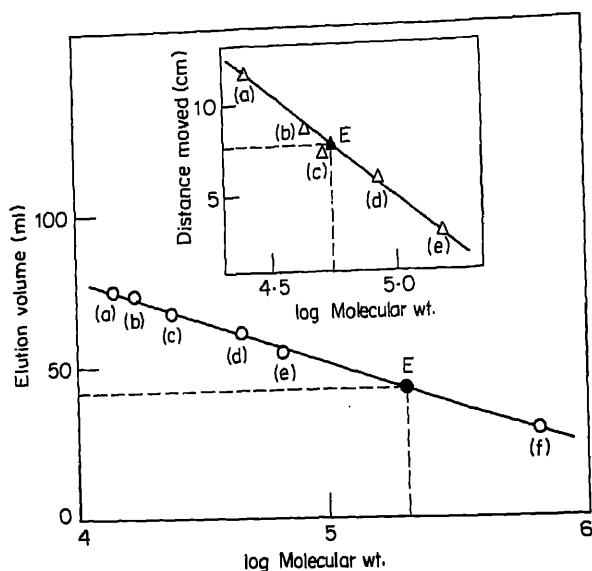
Figure 3. Determination of molecular weight and subunit composition of sheep liver serine hydroxymethyltransferase. For the determination of the molecular weight, the enzyme (E) was passed through a Sephadex G-200 column. The standard protein markers used were lysozyme, (a); myoglobin, (b); chymotrypsin, (c); ovalbumin, (d); bovine serum albumin, (e); and bovine type I thyroglobulin, (f). The elution volumes were plotted as a function of log molecular weight. The molecular weight of serine hydroxymethyltransferase was determined by extrapolation to be 210,000 ±5000. Inset represents sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis in 7.5% gel. The sheep liver enzyme (200 μg) and the marker proteins (200 μg) were separately subjected to denaturation by sodium dodecylsulphate as described above. Another sample of the enzyme and IgG were separately denatured in the presence of sodium dodecylsulphate and 2-mercaptoethanol (2%). The electrophoresis, staining and destaining were done as described in figure 1. The mobilities of α-chymotrypsin, (a); L-chain of IgG, (a); ovalbumin, (b); H-chain of IgG, (c); conalbumin, (d); and undissociated IgG, (e); were plotted as a function of log molecular weight. The subunit molecular weight of the enzyme (E) was determined by extrapolation to be 55,000 ±5000.

Catalytic properties of the enzyme

The specific activity of the enzyme isolated by the above procedure was 6 units/mg protein. Assuming the existence of four identical catalytic sites, the catalytic centre activity was calculated to be 315 min^{-1}. The enzymatic activity increased linearly with the concentration of the enzyme upto about 18 μg/ml reaction mixture. The time course of the enzymatic reaction was linear for over 30 min. The activity of the enzyme increased with temperature upto 60°C and decreased sharply between 60 and 70°C; the activation energy was calculated to be 9.64 Kcal/mol.

Regulatory properties

The enzyme showed positive homotropic cooperative interaction with tetrahydro-folate, giving rise to a sigmoid saturation pattern when preincubated with different concentrations of H_4-folate (figure 4); a Hill coefficient (n_H) value of 2.8 and a $K_{0.5}$
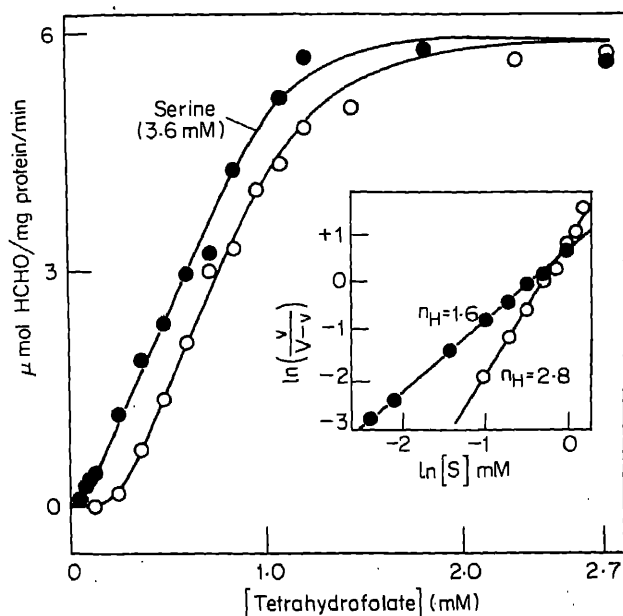
Figure 4. The tetrahydrofolate saturation pattern of the enzyme.

The enzyme (0.6 µg/100 µl) was preincubated for 5 min at 37°C with various concentrations of H_4-folate (O) or L-serine (3.6 mM) (●) and the reaction was started by the addition of serine or tetrahydrofolate. The inset shows a Hill plot, from the slop of which n_H values of 2.8 and 1.6 for the enzyme preincubated with H_4-folate and serine, respectively were obtained.

value of 0.7 mM were calculated from the Hill plot (inset figure 4). The n_H value of 2.8 was seen at all temperatures between 37°C and 60°C. However, this sigmoidicity was reduced to n_H=1.6−1.8 when the enzyme was preincubated for 5 min with a saturating concentration (3.6 mM) of L-serine and the reaction started with varying concentrations of tetrahydrofolate (figure 4). The value did not reach 1.0 as expected for complete loss of homotropic cooperativity in the presence of a positive allosteric effector. This effect of serine was reversed upon dialyzing out the serine. The saturation pattern of L-serine was hyperbolic with a K_m value of 0.9 mM (figure not given). NAD^+ and NADH were negative and positive allosteric effectors with respect to tetrahydrofolate saturation (figures 5 and 6). NAD^+ (2 mM) increased the sigmoidicity to an n_H of 3.3 and at 10 mM the n_H value reached 3.8, very close to the theoretical maximum (n_H=4) for an enzyme with four subunits (inset figure 5). NADH decreased the cooperativity to n_H=2 at 5 mM (not shown in the figure) and to n_H1.8 at 50 mM (inset figure 6).

In a recent communication Schirch and Quashnock (1981) have attributed the cooperativity observed by us earlier with the monkey liver and mung bean enzymes (Ramesh and Appaji Rao, 1978; 1980a, b; Rao and Appaji Rao, 1980) to the oxidation of tetrahydrofolate, during preincubation and assay, and have remarked that the sigmoidicity could be dependent on the time of preincubation of the enzyme with H_4-folate and the assay conditions. In order to resolve this problem, the oxidation of tetrahydrofolate was examined by spectral, chemical and
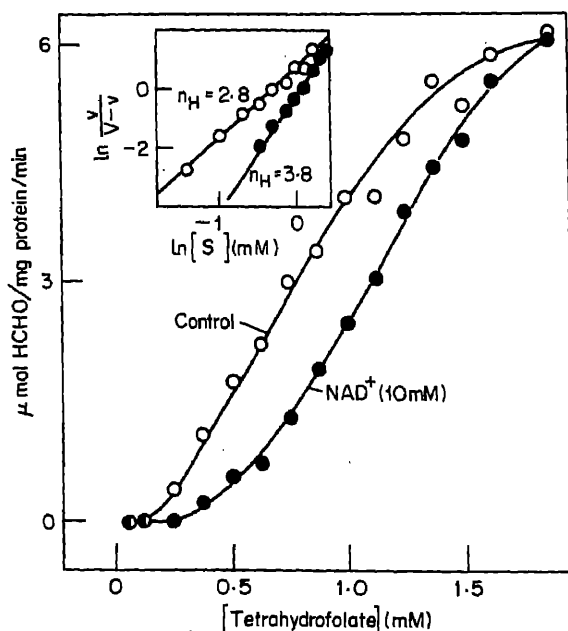
Figure 5. The effect of NAD$^+$ (10 mM) on the H$_4$-folate saturation pattern.

The enzyme (0.6 μg/100 μl) was preincubated with various concentrations of H$_4$-folate (O) and also with H$_4$-folate and NAD$^+$ (10 mM) (●) for 5 min at 37°C and the reaction was started by the addition of L-serine (3.6 mM). The inset shows a Hill plot, from the slope of which n_H values of 2.8 and 3.8 were obtained in the absence and presence, respectively of NAD$^+$
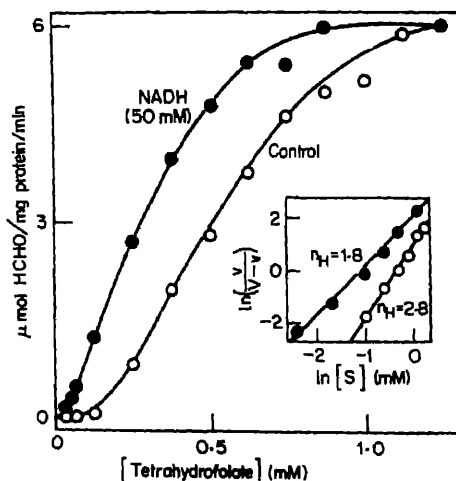
Figure 6. The effect of NADH (50 mM) on the H$_4$-folate saturation pattern.

The enzyme (0.6 μg/100 μl) was preincubated with various concentrations of H$_4$-folate (O) and also with H$_4$-folate and NADH (50 mM) (●) for 5 min at 37°C and the reaction was started by the addition of L-serine (3.6 mM). The inset shows a Hill plot, from the slope of which n_H values of 2.8 and 1.8 were obtained for the absence and presence, respectively of NADH.

enzymatic methods. The tetrahydrofolate prepared by us by the procedure of Hatefi *et al.* (1959) and the commercially available samples from Sigma Chemical Co. were incubated at 37°C in phosphate buffer (pH 7.4) containing 1.8 mM dithiothreitol for time periods of 0, 10, 20, 40 and 80 min. Even at the lowest concentration of H_4-folate used by us for the assay of serine hydroxymethyltransferase, only 5% oxidation was observed. The spectral change was minimal as recorded at the time intervals mentioned in a Gilford recording spectrophotometer. It can be seen from table 2 that very little oxidation seems to have occurred when H_4-folate was incubated at 37°C for periods of time ranging from 0 to 20 min and estimated by a chemical method (Huennekens *et al.*, 1963).

Table 2. Stability of H_4-folate during preincubation and assay of serine hydroxymethyltransferase.

Time (min)	Chemical assay (μmol N^5, N^{10}-methenyl-H_4-folate)	Enzymatic assay (μmol HCHO)
0	0.025(100)	0.025(100)
10	0.025(100)	0.024(96)
20	0.024(96)	0.023(92)
40	0.022(88)	0.021(84)

* Numbers in parentheses indicate per cent of control (0 min) value.

H_4-folate (0.25 mM) was incubated at 37°C and aliquots were drawn at 0, 10, 20, 40 and 80 min, and the amount of H_4-folate present was estimated by converting it completely to N^5, N^{10}-CH_2-H_4-folate by adding excess of enzyme (60 μg, compared to 0.6 μg normally used in the assay) and labelled serine. It can be seen from table 2 that very little H_4-folate was oxidized in 20 min, the time taken for preincubation and assay of the serine hydroxymethyltransferase.

The substrate saturation patterns at varying concentrations of H_4-folate and at a saturating concentration of serine (3.6 mM) were carried out by preincubating the enzyme with tetrahydrofolate and assaying the enzyme activity for 1, 5 and 15 min. The n_H values determined from the Hill plots were 2.2, 2.8 and 2.8 respectively. The requirement for preincubation could be due to the slow conformational change induced by the binding of these ligands. However, this needs to be carefully examined.

Inhibition by folate and serine analogues

Table 3 gives the per cent inhibition of the activity of the enzyme at various concentrations of folate and serine analogues added to the standard reaction mixture. Dichloromethotrexate was a potent inhibitor of the enzyme activity, inhibiting it 87% at a concentration of 20 mM, whereas aminopterin only produced 35% inhibition at this concentration. Methotrexate was without effect at this

Table 3. Inhibition of sheep liver serine hydroxymethyltransferase by folate and serine analogues.

Inhibitor	Concentration (mM)			
	1	5	10	20
	PER CENT INHIBITION			
Folate	10	38	53	63
Dihydrofolate	7	24	35	45
Aminopterin	5	18	27	35
Dichloromethotrexate	35	59	74	87
O-Acetyl-L-serine	3	11	21	38
Phospho-L-serine	10	26	37	43
L-O-Methylserine	23	50	63	71

concentration. Folate and dihydrofolate were not very strong inhibitors of th sheep liver serine hydroxymethyltransferse. The inhibition produced by serir analogues were in the following order of effectiveness: O-methylserine >phosph serine >O-acetylserine. Figures 7a and 7b show double reciprocal plots
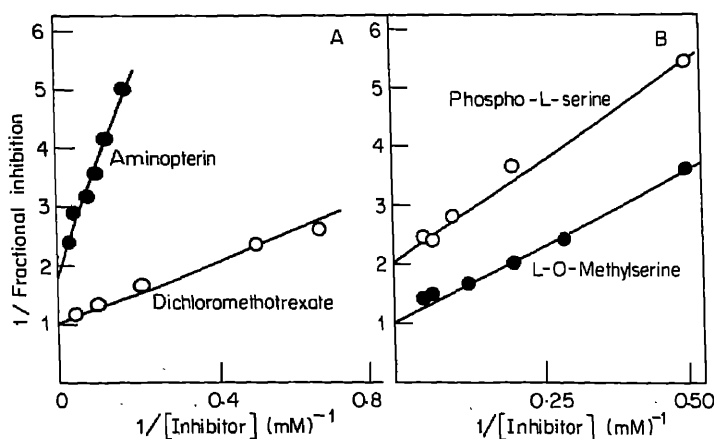
Figure 7. Partial inhibition analysis of the interaction of sheep liver serinehydroxymethyl-transferase with aminopterin (●), dichloromethotrexate (O) (figure 7a) and phospho-L-serine (O), L-O-methylserine (●) (figure 7b). The enzyme (0.6 µg/100 µl) was preincubated for 5 min at 37°C with H_4-folate (1.8 mM) and the inhibitor. The enzyme activity in the absence of inhibitor was normalized to 1. The theoretical basis for this analysis is as follows:

$$\frac{1}{i} = \frac{\alpha K_i(K_m + [S])}{K_m(\alpha - 1)} \cdot \frac{1}{[I]} + \alpha \frac{\alpha K_m + [S]}{K_m(\alpha - 1)} \quad (1)$$

where $i = 1 - V_i/V$, V_i being the velocity of the reaction in the presence of inhibitor and V being

the velocity in the absence of the inhibitor; u is the factor by which K_i and K_s are altered when EI and ES react with the substrate or inhibitor. The ordinate intercept in a plot of $1/i$ vs $1/[I]$ is given by:

$$\frac{aK_m+|S|}{K_m(a-1)} \qquad\qquad \text{When } |I|=\infty, \; 1/i=|K_i(1+\frac{|S|}{K_m}|1/|I|+1.]$$

Thus, for a complete competitive inhibitor the ordinate intercept is 1 in the above plot and >1 for a partial competitive inhibitor.

fractional inhibition versus the concentration of inhibitor. Folic acid and dichloromethotrexate were complete inhibitors while dihydrofolate and amino-pterin were partial inhibitors. Phospho L-serine was a partial inhibitor while DL-O-methylserine and O-acetyl L-serine were complete inhibitors. β-Phenylserine and threonine had no effect on the enzyme activity at these concentrations. Figure 8 shows the inhibition by D-cycloserine; the inhibition was sigmoid and the inhibition at 1.0, 1.5 and 1.75 mM were significantly less (0, 20 and 47% compared to 12, 43 and 65%) when the enzyme was preincubated with L-serine instead of with tetrahydrofolate (figure 8).
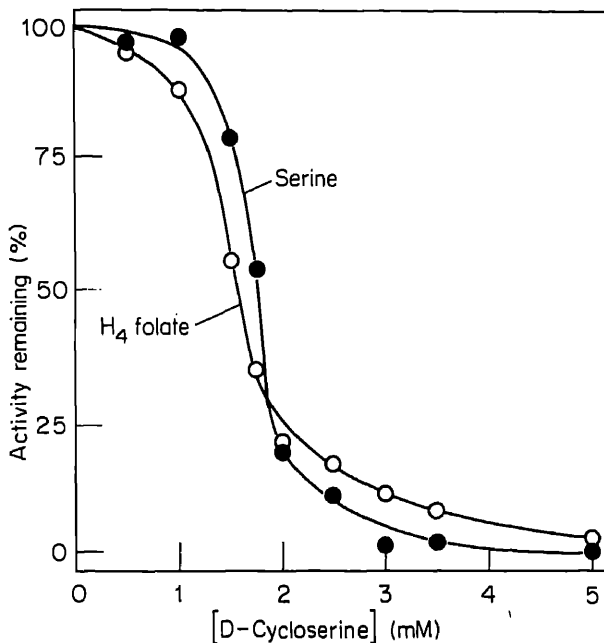
Figure 8. Inhibition of sheep liver serine hydroxymethyltransferase by D-cycloserine.

The enzyme (0.6 μg/100 μl) was preincubated for 5 min at 37°C with D-cycloserine and H_4-folate (1.8 mM) (O) or L-serine (3.6 mM) (●) before initiating the reaction by the addition of the second substrate.

Inhibition by Cibacron Blue

Cibacron blue inhibited the enzyme activity completely (figure 9). NAD (30 mM) partially reversed the inhibition caused by Cibacron blue at all concentrations of the dye as shown in figure 9. Increasing concentrations of NAD+ at a fixed
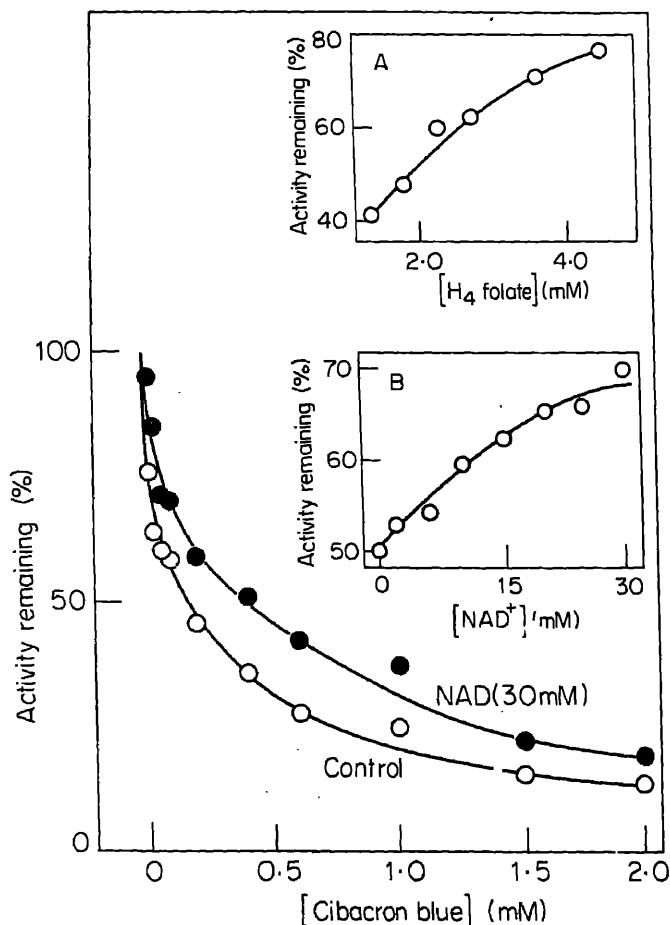
Figure 9. The inhibition pattern of sheep liver serinehydroxymethyltransferase by Cibacron blue.

The inhibition is measured in the absence (O) and in the presence (●) of NAD+. The enzyme (0.8 μg/100 μl) was preincubated for 5 min at 37°C with different concentrations of the dye and with H$_4$-folate (1.8 mM) alone or with H$_4$-folate (1.8 mM) and NAD+ (30 mM) and the reaction started by the addition to L-serine (3.6 mM). Inset A shows a plot of the enzyme activity as per cent of control (activity in the absence of any inhibitor and at saturating concentrations of substrates 3.6 mM serine and 1.8 mM H$_4$-folate) vs varying concentration of H$_4$-folate (1.8 to 5.5 mM) (O). Inset B shows a plot of the enzyme activity as per cent of control (in the presence of 0.2 mM Cibacron blue and at saturating concentrations of H$_4$-folate and L-serine) vs concentration of NAD+ (0 to 30 mM). NAD+ (30 mM) did not alter the activity of the control. The concentration of Cibacron blue was fixed at 0.2 mM in all these experiments and inhibited the enzyme activity to 40% of the control value.

concentration of Cibacron blue (0.2 mM) caused increasing reversal of inhibition (figure 9, inset B). Similarly, tetrahydrofolate could also overcome the inhibition by Cibacron blue (figure 9, inset A). Double reciprocal plots of the data showed that at infinite concentration of NAD$^+$, reversal of the inhibition to 80% of the uninhibited activity was obtained whereas with H$_4$-folate complete reversal of the inhibition was reached (figures not given).

Heat stability of the enzyme

It was earlier observed that heat inactivation in the presence of L-serine was a convenient method to purify the enzyme (Schirch, 1971) but it was shown by us that this procedure resulted in the desensitization of the monkey liver enzyme (Ramesh and Appaji Rao, 1980a). It was, therefore, of interest to study the effect of heat on the enzyme in the presence of substrates. Figure 10 shows the heat inactivation
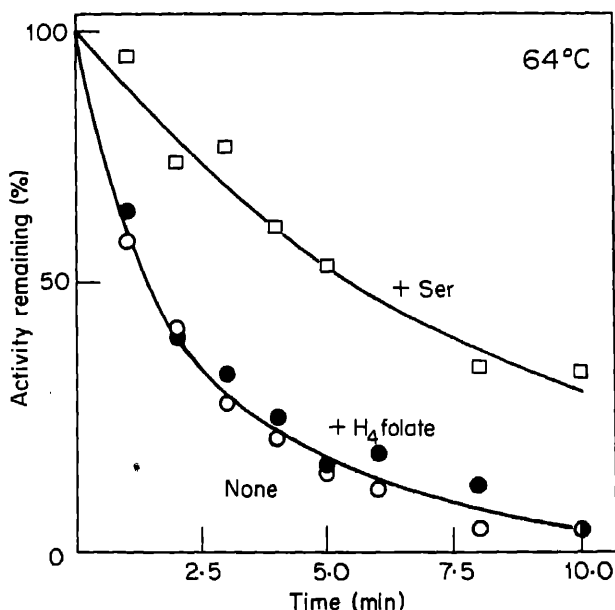
Figure 10. The thermal denaturation profiles of sheep liver serinehydroxymethyltransferase. (a) In the presence of L-serine (3.6 mM) (□), (b) H$_4$folate (3.6 mM) (●) and (c) in the absence of any substrate (O). The enzyme was incubated (with or without substracte) at 64°C and a constant volume pipetted out at every time point into an assay tube which was immediately chilled in ice. At the end of 10 min the aliquots of the enzyme were assayed at 37°C at saturating concentrations of H$_4$-folate and L-serine Since H$_4$-folate was easily oxidized, 3.6 mM H$_4$-folate alone was taken in a separate experiment, kept at 64°C for 10 min and assayed enzymatically at 37°C in order to ensure that it did not decrease below the saturating concentration in the above experiment.

patterns at 64°C of the native enzyme, the enzyme in the presence of a saturating concentration (3.6 mM) of L-serine and the enzyme in the presence of a saturating concentration of H$_4$-folate. It is seen from the figure that the heat inactivation patterns of the native enzyme and the enzyme in the presence of H$_4$-folate are similar but differ significantly from that of the enzyme in the presence of serine.

Spectroscopic characterization

The enzyme had two absorption maxima at 277 and 428 nm. The enzyme showed two fluorescence emission maxima at 340 nm and 337 nm when excited at 285 nm and 295 nm, respectively (figures not given). The far u.v., c.d. spectrum (figure 11) was characteristic of a protein containing a large amount of α-helix (33%); the $[\theta]_{mrw}$ values at 200 and 208 nm were 11.45 and 11.66×10^3 deg cm^2 d mol^{-1}. The visible and near u.v., c.d. spectrum (figure 12) showed a peak at 430 nm characteristic of bound pyridoxal 5′ phosphate and negative peaks at 268 and 27 nm, characteristics of aromatic amino acid residues.
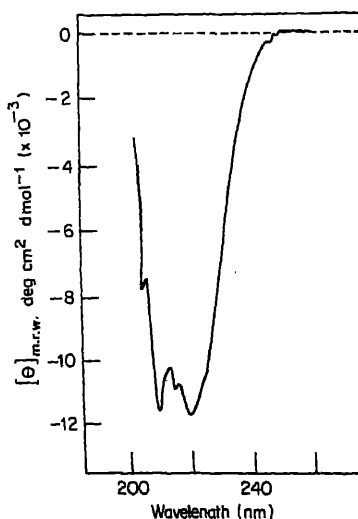
Figure 11.   The far uv c.d. spectrum of sheep liver serine hydroxymethyltransferase.

The spectrum was taken at an enzyme concentration of 0.3 mg/ml in a 1 mm cell, after dialysing out free pyridoxal 5′ phosphate.
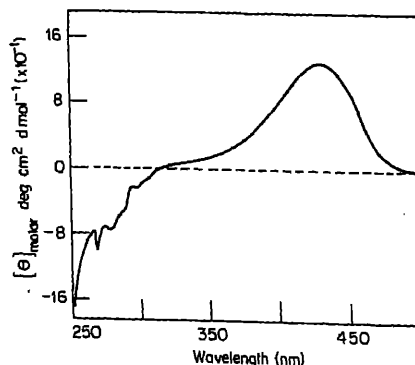
Figure 12.   The near u.v. and visible c.d. spectrum of sheep liver serine hydroxymethyltransferase.

The spectrum was taken at a protein concentration of 0.885 mg/ml in a 10 mm cell, after dialysing out free pyridoxal 5′ phosphate.

Discussion

The kinetic and catalytic reaction mechanisms of serine hydroxymethyltransferase have been studied extensively albeit not elucidated completely with the enzyme obtained from several mammalian and microbial sources (Schirch *et al.,* 1973; Ching and Kallen, 1979; Hanson and Davis, 1979; Ulevitch and Kallen, 1977a, b). Except for the observations on the enzyme from pig kidney (Harish Kumar *et al.,* 1976) and monkey liver (Ramesh and Appaji Rao, 1978, 1980a), there is very little information on its regulation. The sheep liver enzyme, isolated in a homogeneous form (figures 1, 2 and 3) by avoiding the use of heat denaturation in the presence of L-serine during the purification resulted in the retention of its allosteric properties. The sheep liver enzyme purified by us was different from the monkey, rabbit, lamb and rat enzymes (Schirch, 1971; Nakano *et al.,* 1968; Ulevitch and Kallen, 1977a; Ramesh and Appaji Rao, 1980a) with respect to several important features, although there was essential similarity in the physicochemical properties of these enzymes.

The sheep liver enzyme, like the enzyme isolated from other sources was adsorbed strongly to CM-Sephadex and to folate-Sepharose. Another characteristic feature shared by this enzyme with the pig kidney (Braman *et al.,* 1981) and monkey liver enzymes (Ramesh and Appaji Rao, 1980a) is its ability to interact with Cibacron.Blue-Sepharose. The pH and temperature optima of this enzyme were similar to those of the other enzymes. All the serine hydroxymethyltransferases isolated so far have a molecular weight of about 200,000 and are made up of four identical single polypeptide subunits (Fujioka, 1969; Ulevitch and Kallen, 1977a; Ramesh and Appaji Rao, 1980a; Rao and Appaji Rao, 1980). All these enzymes except that from the mung bean seedlings (Rao and Appaji Rao, 1981) require pyridoxal 5' phosphate for catalysis and sulphydryl groups for activity.

Unlike the enzyme from rabbit, lamb, ox etc. (Jones and Priest, 1976; Ulevitch and Kallen, 1977a, b), the sheep liver serine hydroxymethyltransferase exhibits homotropic cooperative interactions with the substrate, H_4-folate, as evidenced by a sigmoid saturation pattern (figure 4) and an n_H value of 2.8. The pig kidney (Harish Kumar *et al.,* 1976) and monkey liver (Ramesh and Appaji Rao, 1978) enzymes also show this cooperativity with n_H values of 3.9 and 2.5, respectively. The cooperativity of H_4-folate interactions was abolished in the monkey liver enzyme by preincubation with a saturating concentration (3.6 mM) of L-serine (Ramesh and Appaji Rao, 1978), whereas this treatment only decreased the n_H value of the sheep liver enzyme to 1.6-1.8 (figure 4), suggesting that the nature of cooperative interactions in this enzyme was different. NADH, a positive allosteric effector for the monkey liver enzyme (Ramesh and Appaji Rao, 1978) converted the sigmoid saturation pattern to hyperbolic ($n_H=1$) whereas in the sheep liver enzyme, NADH could only reduce the n_H value to 1.8 (figure 6). NAD^+ was a negative allosteric effector in both the cases (figure 5). In the monkey liver enzyme, cooperativity was abolished at 60°C whereas an n_H value of 2.8 was observed in the sheep liver enzyme even at 60°C. On the contrary, and unlike the monkey liver enzyme, after several months of storage at −40°C, the sheep liver enzyme showed decreased cooperativity ($n_H=1.8$) along with decreased (about 30% less) activity (results not shown).

Schirch and Quashnock (1981) have concluded that there is no cooperativity in the interactions of tetrahydrofolate with serinehydroxymethyltransferase based on the observation that tetrahydrofolate is oxidized at low concentrations (below 20 µM) by a complex second order process dependent on the concentrations of tetrahydrofolate and oxygen. They have also observed that sigmoidicity was dependent on the time of preincubation and assay. Using a coupled assay and measuring the velocity within 20 seconds, they have obtained a hyperbolic saturation pattern which was different from the sigmoid pattern obtained using the assay procedure employed by us. They were also unable to obtain any NADH effects and failed to observe binding of NADH to the ·enzyme by equilibrium dialysis.

Our results with the sheep liver enzyme reported in this communication are contrary to these observations. The discrepancy is apparently due to their using tetrahydrofolate concentrations which were an order of magnitude less than those used by us (0.04-0.25 mM by Schirch and Quashnock (1981), and 0.25-1.8 mM by us in this study and by Ramesh and Appaji Rao, 1978,(1980a, b) suggesting that two tetrahydrofolate saturation patterns may exist in this enzyme. The discrepancy between our results and those of Schirch and Quashnock (1981) could not be due to oxidation of tetrahydrofolate as at the concentrations used by us, no oxidation of tetrahydrofolate was observed (table 2). The hypothesis put forth by Schirch and Quashnock (1981) that the observed cooperativity is due to an artifact of the assay and not an inherent property of the enzyme, appears invalid, as the n_H values for different enzyme preparations (3.9, 1.0, 2.5, 2.2 and 2.8, respectively for the pig kidney, mouse L1210 tumor, monkey liver, mung bean and sheep liver enzymes) (Harish Kumar *et al.,* 1976, Ramesh and Appaji Rao, 1978; Rao and Appaji Rao, 1980) vary considerably and denaturation with heat results in the alteration of n_H values for the monkey liver enzyme (Ramesh *et al.,* 1981b) but not for the mung bean (Rao and Appaji Rao, 1980) and sheep liver enzymes. In addition, there is specificity in the interaction of effector molecules; some, like NADH, decrease the cooperativity and others, like NAD^+, increase the cooperativity. These observations clearly point out that cooperativity of H_4-folate interactions is an inherent property of the enzyme rather than an artifact of assay.

Although Schirch and Quashnock (1981) report their inability to observe NADH effects of the kinetics as well as binding of this ligand to the monkey liver enzyme, we have observed in this study that pyridine nucleotides alter the cooperativity of the tetrahydrofolate interactions with the sheep liver enzyme and also affect the inhibition of the enzyme by Cibacron Blue. These workers, based on the binding studies of the monkey liver enzyme with NADH, remark that 'if binding is occurring, it may be taking place with a Kd value greater than 2 mM'. It can be seen from the results presented in this study that the minimum concentration of NADH required to cause a significant change in the n_H value is approximately 5 mM. Therefore, it is not surprising that binding was not observed when the equilibrating chambers contained 0.5 mM NADH (Schirch and Quashnock, 1981).

The antibiotic, D-cycloserine, was a potent linear competitive inhibitor of the monkey liver enzyme (Ramesh, 1980), whereas it inhibited the sheep liver enzyme

in a sigmoid pattern (figure 8). The above observations point to significant differences in the quaternary structure and subunit interactions of the monkey and sheep liver enzymes. Antibodies raised against the monkey liver enzyme partially cross-reacted with the sheep liver enzyme and *vice versa* (Ramesh and Appaji Rao, 1980a), suggesting some similarity in the structures of these two proteins but also indicating that they were not identical. This preliminary investigation seems to suggest that a comparative study of the enzyme from these two sources involving an analysis of their desensitization and denaturation profiles, is essential for the understanding of a common basis for subunit interactions among the serine hydroxymethyltransferases.

A comparison of the catalytic properties of the sheep liver enzyme with that from other sources shows some common features and several characteristic differences. The V_{max} of the monkey and sheep liver enzymes were 3.3 and 6 units, respectively; the $K_{0.5}$ values of these two enzymes were 0.54 and 0.7 mM, respectively for tetrahydrofolate and 0.74 mM and 0.9 mM, respectively for L-serine. Unlike the enzyme isolated from other sources (Ulevitch and Kallen, 1977a, b, c), β-phenylserine (20 mM) and threonine (100 mM) did not inhibit significantly the sheep liver enzyme. A possible reason for this difference could be the use of a desensitized enzyme by other workers.

Dichloromethotrexate inhibited the sheep liver enzyme completely, whereas aminopterin was a partial inhibitor (figure 7); both these antifolates were complete inhibitors of the monkey liver enzyme (Ramesh and Appaji Rao, 1980a). Methotrexate, on the other hand, was a partial inhibitor of the monkey liver enzyme while it did not produce significant inhibition of the sheep liver enzyme. Folate was a complete inhibitor of both the enzymes while dihydrofolate was a partial inhibitor.

Cibacron Blue has been used to probe into the 'nucleotide-fold' of dehydrogenases, kinases and several binding proteins (Rossman *et al.*, 1974; Thompson *et al.*, 1975; Thompson and Stellwagen, 1976; Stellwagen, 1977; Lepo *et al.*, 1979), but recent evidence seems to suggest that it might interact at other sites on the enzyme, especially at hydrophobic pockets (Subramanian and Kaufman, 1980; Grazi *et al.*, 1978; Beissner *et al.*, 1979; Barden *et al.*, 1980). Interaction of the dye with the monkey liver serine hydroxymethyltransferase is a clearcut instance of binding at the folate domain due to structural similarity in the triazine moiety of the dye and folic acid (Ramesh and Appaji Rao, 1980b). The interaction of triazines at the active site of this enzyme is indicated by the inhibition of the activity of the pig kidney enzyme by the Baker antifolates (Harish Kumar, P. M., Appaji Rao, N. and Mangum, J. H.—personal communication). In the case of the sheep liver enzyme, however, NAD$^+$ and NADH as also tetrahydrofolate, are able to overcome significantly the inhibition by the dye, indicating multiple interacting sites for the dye with this enzyme.

The results presented in this paper clearly demonstrate that serine hydroxymethyltransferase is a regulatory protein and that the sheep liver enzyme could be important system to probe into the structure, function and regulation of this first enzyme of the folate pathway.

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Immobilization of trypsin on sand: Mode of binding

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Abstract. Acid-washed and heat-treated river sand was separated into different fractions by geochemical methods and immobilization of trypsin was carried out on the separated fractions using 3-aminopropyltriethoxysilane and glutaraldehyde. Scanning Electron Micrographs of the purified fraction (Sp. gr >2.5 and <2.8) of magnetically non-susceptible sand and quartz showed that the enzyme could be fixed on the supports. Malonic acid (16.3 nmol and 16.7 nmol per *g*) appeared to be bound to alkylamine purified fraction of magnetically non-susceptible sand and alkylamine quartz, respectively. Studies on the effect of 6 M guanidine.HCl on immobilized trypsin demonstrated that immobilized trypsin had considerable stability against denaturation. The results obtained indicated that magnetically non-susceptible sand was found to be nearly as good as quartz for trypsin immobilization and that trypsin was covalently coupled to sand via 3-aminopropyltriethoxysilane and glutaraldehyde.

Keywords. Sand; 3-aminopropyltriethoxysilane; trypsin; quartz; scanning electron microscopy.

Introduction

A large number of enzymes have been immobilized on inorganic carriers like porous glass (Adamich *et al.*, 1978), ceramics (Dale and White, 1979), carbon (Cho and Bailey, 1978) and sand (Puls *et al.*, 1977) by different techniques. Trypsin has been immobilized on sand by different coupling methods and maximum activity retained was observed for the enzyme immobilized on sand-3-aminopropyltriethoxysilane (APTS) compound (alkylamine sand) by coupling through glutaraldehyde (Puvanakrishnan and Bose, 1980a).

In the present study, in order to find out the possible binding sites for APTS on sand, the following parameters were investigated: (a) the geochemical separation of sand into different fractions and immobilization of trypsin on the separated fractions in comparison with immobilization on pure quartz; (b) scanning electron microscopy studies of both the purified fraction (Sp. gr. >2.5 and <2.8) of magnetically non-susceptible sand (PFMNS) and quartz at every stage of the preparation of immobilized enzyme; (c) determination of potentially reactive sites

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present on the surfaces of both the PFMNS-APTS compound (alkylamine PFMN
and quartz-APTS compound (alkylamine quartz); and (d) the effect of 6
guanidine.HCl on the immobilized trypsin.

Materials and methods

Materials

Trypsin from M/s. E. Merck, glutaraldehyde (25% solution) from M/s. Ried
Germany, 1-ethyl-3-dimethyl-aminopropyl) carbodiimide HCl (EDC) from M
Sigma Chem. Co., St. Louis, Missouri, USA, APTS from M/s. E. Merck, Germa
and [2−¹⁴C] malonic acid from M/s. New England Nuclear Corporation, Bost
Massachussets, USA were used in the present study. Quartz was collected from
pure quartz-vein.

Preparation of sand and quartz for use as carriers

River sand was sieved to collect the required particle size (44 mesh ~355 µ
After coning and quartering, the collected sand was soaked in concentrated nit
acid (A.R. grade) for a day and washed thoroughly to remove all acid. It was th
dried in an air oven at 100°C, heated in a muffle furnace at 500°C for 6 h, coo
and stored. Quartz was also prepared in the same way.

Fractionation of sand

Acid washed sand, prepared as above, was separated into two fractions
magnetically non-susceptible sand and magnetically susceptible sand by pass
through Isodynamic separator (Chas, W. Cook and Sons Ltd., UK). These t
fractions were heated at 500°C for 6 h, cooled and separately used for immobili
tion of trypsin.

Magnetically non-susceptible sand was further concentrated on the basis
specific gravity using bromoform solution (Milner, 1952). The purified fract
(Sp. gr. >2.5 and <2.8) was collected, washed thoroughly with benzene, heate
500°C for 6 h, cooled and then used for trypsin immobilization.

Immobilization of trypsin on sand and quartz

Sand, as prepared above, was silanized using APTS and trypsin was bound to
alkylamine sand using glutaraldehyde (Puvanakrishnan and Bose, 1980a). ⸴
same procedure was followed for the immobilization of trypsin on quartz (44 m
particle size ~355 nm).

Estimation of proteolytic activity

Proteolytic activity of immobilized trypsin was assayed by Anson's colorime
method (Anson, 1938) with suitable modifications (Puvanakrishnan and B
1980a). One unit of enzyme activity is defined as one µg of tyrosine liberated fi
15 ml of digestion mixture at 45°C in 30 min. The specific activity is calculate
units of enzyme activity per mg of protein.

Analysis of immobilized trypsin

Protein bound to sand was estimated by the micro-kjeldhal method and was fo
out by determining the difference between the initial enzyme protein in solu

and total enzyme protein recovered after coupling and washing. The retained activity per cent of immobilized trypsin was calculated from the specific activities of native and immobilized trypsin. Glutaraldehyde bound to sand was estimated using N-methylbenzethiazolone hydrazone (Paz *et al.,* 1965) by determining the difference between the initial gluaraldehyde in solution and the glutaraldehyde recovered after coupling and washing and was expressed in terms of the amount of acetaldehyde.

Chemical analysis of sand fractions and quartz

Representative samples of sand, magnetically susceptible or non-susceptible sand, purified fraction (Sp. gr. >2.5 and <2.8) of magnetically non-susceptible sand (PFMNS) and quartz were analysed to determine the percentage composition of the constituents viz. SiO_2, Fe_2O_3, Al_2O_3 and MgO (Jeffery, 1970).

Scanning Electron Microscopy of PFMNS and quartz

Representative samples of PFMNS and quartz were prepared for examination in a Scanning Electron Microscope, Stereoscan S 150, operated at an accelerating voltage of 10 KV (Johari and De-Nee, 1972). The Scanning Electron Micrographs shown in the present investigation were obtained at a magnification of 2000 X.

Potential reactive sites (reactive amine groups)

Potential reactive sites on alkylamine PFMNS and alkylamine quartz were quantitated by coupling with [2-^{14}C] malonic acid in the presence of 3-(3-dimethyl aminopropyl)-1-ethylcarbodiimide (EDC) and the radioactivity bound was measured at an interval of 60 min for a total period of 300 min (Brotherton *et al.,* 1976). The radioactive samples were counted in Packard PRIAS liquid scintillation system.

Stability toward 6 M guanidine.HCl

Trypsin immobilized on sand (20 g) was soaked in 100 ml of 6 M guanidine. HCl, pH 8.0, at room temperature for 45 min and the activity was assayed. This procedure was repeated five times using fresh 6 M guanidine.HCl for each soak.

Results and discussion

River sand usually contains different constituents like quartz, feldspars, olivines, micas, amphiboles, pyroxenes, other common silicates etc. All iron-bearing minerals were removed when sand was passed through Isodynamic separator, thus resulting in two fractions viz. magnetically non-susceptible sand and magnetically susceptible sand. Magnetically non-susceptible sand was further concentrated to a fraction having Sp.gr >2.5 and <2.8 using bromoform since the purest quartz (rock crystal) has a Sp.gr. of 2.69, while other forms range from 2.5 to 2.8 (Dake *et al.,* 1938).

It is seen from table 1 that trypsin immobilized on magnetically non-susceptible sand gave a higher bound activity than trypsin immobilized on magnetically susceptible sand. This might probably be due to the higher silica (SiO_2) content present in the magnetically non-susceptible sand as shown by the chemical analysis data (table 2). However, significant rise in the retained activity level could not be

noticed when trypsin was immobilized on PFMNS (table 1). The results o
content (table 2) lent support to these findings.

Table 1.   Analysis of immobilized trypsin

Carrier	Specific activity	Protein bound mg/g	Retained activity (%)	Glutaraldehyd bound express as acetaldehyc mg/g
Sand	624	1.88	94.5	1.99
Magnetically susceptible sand	519	1.56	78.6	1.66
Magnetically non-susceptible sand	632	2.03	95.8	2.10
Purified fraction (Sp.gr. > 2.5 and < 2.8) of magnetically non-susceptible sand	636	2.27	96.3	2.23
Quartz	643	2.31	97.3	2.30

Table 2.   Chemical analysis of sand fractions and quartz

Carrier	SiO_2 (%)	Fe_2O_3 (%)	Al_2O_3 (%)	MgO (%)
Sand	94.65	1.72	3.02	0.69
Magnetically susceptible sand	89.1	5.12	4.93	0.89
Magnetically non-susceptible sand	97.00	0.96	1.53	0.51
Purified fraction (Sp.gr. > 2.5 and < 2.8) of magnetically non-susceptible sand	99.53	0.18	0.07	0.10
Quartz	99.82	—	—	—

When trypsin was bound to magnetically susceptible sand and quartz respe
(table 1) 1.56 mg protein/g and 2.31 mg protein/g were observed. The low l
protein binding to sand might be due to lower effective surface area and e
loading capacity (Olson and Korus, 1977) as well as to the nonporous nature
sand material (Puls *et al.*, 1977). A similar result of low protein binding as ob:
here had been noticed when glucose oxidase was immobilized on nickel ox
nickel screens (Weetall and Hersh, 1970). The glutaraldehyde bound t
magnetically non-susceptible sand and PFMNS showed only a slight incre
compared to sand and the data obtained on the protein bound also show
same trend.

Most of the inorganic materials used as supports are known to have hydroxyl and oxide groups on their surfaces (Lynn, 1977; Weetall and Detar, 1975). The probable involvement of the alkoxy or silanol end of APTS with the surface hydroxyl groups of inorganic surfaces was suggested earlier (Pleuddemann, 1970). The two inorganic materials, sand and its purest form viz. quartz were studied comparatively as carriers for trypsin immobilization to find out whether hydroxyl groups are the possible binding sites for APTS on sand. Quartz, the purest form of sand, was reported to have predominantly −OH group on its surface (Vanderbilt and Jaruzelski, 1962). The results obtained in this study showed that the retained activities of trypsin immobilized on sand and quartz were nearly the same which indicated that the mechanism of binding of APTS was to a great extent dependent on the hydroxyl groups on the surface of sand.

Studies by Scanning Electro Microscpy of both PFMNS and quartz were carried out at different stages of preparation of the immobilized enzyme to confirm the nature of binding of trypsin. Figures 1 and 2 show typical Scanning Electron Micrographs of the surface structure of PFMNS and quartz respectively after acid wash and heat treatment. When the acid washed and heat treated PFMNS and quartz were reacted with APTS and gluaraldehyde and subsequently treated with trypsin, the surface features appeared to be masked by deposits (compare figures 3 and 4 with figures 1 and 2 respectively). It was evident from these electron micrographs that the enzyme protein could be fixed on the supports and also that the binding was more distinct on quartz (figure 4) than on PFMNS (figure 3). This might be due to the crystalline nature of quartz.
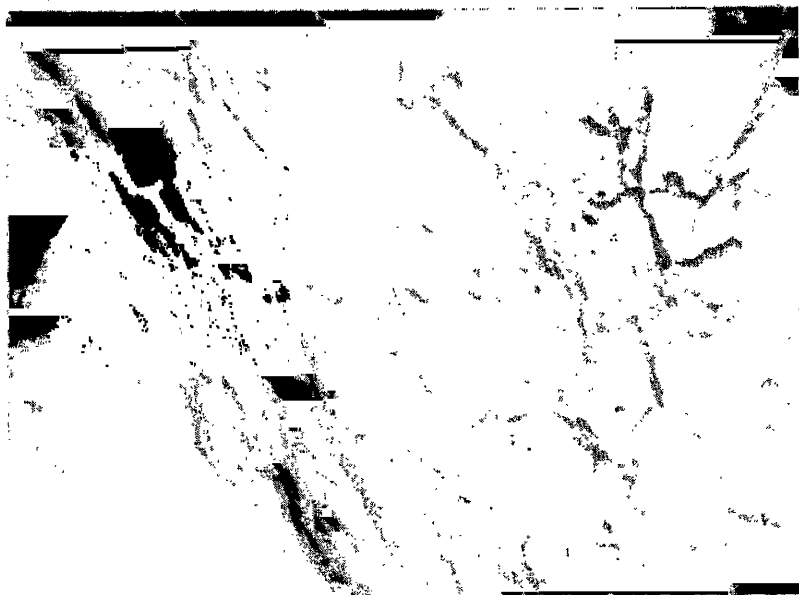
Figure 1. SEM of acid washed and heat treated PFMNS.

Figure 2.   SEM of acid washed and heat treated quartz.
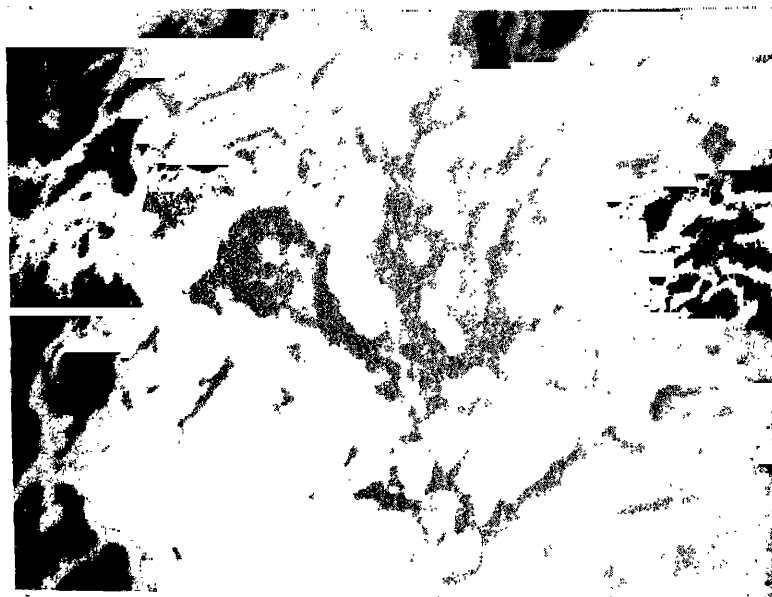
Figure 3.   SEM of acid washed, heat treated and APTS-glutaraldehyde treated PFMNS finally
bound with trypsin.

Figure 4. SEM of acid washed, heat treated and APTS-glutaraldehyde treated quartz finally bound with trypsin.

Earlier studies on potential reactive sites (reactive amine groups) present on the surface of alkylamine sand using [2-^{14}C] malonic acid (Puvanakrishnan and Bose, 1980b) revealed that malonic bound/g was higher (15.5 nmol) for alkylamine sand and much less (4.5 mol) for sand, washed with nitric acid and subjected to heat treatment. The additional amount of malonic acid bound (11 nmol) was suggestive of the increased potential reactive sites imparted by APTS. Comparative studies made on alkylamine quartz and alkylamine PFMNS showed that malonic acid bound was more or less the same viz. 16.7 nmol/g and 16.3 nmol/g for alkylamine quartz and alkylamine PFMNS respectively indicating that the potential reactive sites were almost equally present in these two fractions. Thus, purification of sand to PFMNS would further improve the availability of reactive sites approaching the quality very near to that of quartz. These findings lent support to the view that the alkoxy end of the APTS coupled with the sand hydroxyl groups leaving the reactive amine groups of APTS free for reaction with glutarldehyde.

In figure 5 it was observed that the immobilized trypsin retained almost all its original activity until the second week in the presence of 6 M guanidine. HCl. Under the identical conditions, native trypsin was observed to loose all its activity within 45 min. These results were in general agreement with the earlier studies on the stability of immobilized trypsin toward 6 M urea wherein the immobilized trypsin retained its original activity till the second soak with a decrease only after the third soak (Puvanakrishnan and Bose, 1980b). These findings amply demons-

trated that trypsin was not absorbed to the carrier but was covalently coupl
APTS and glutaraldehyde.
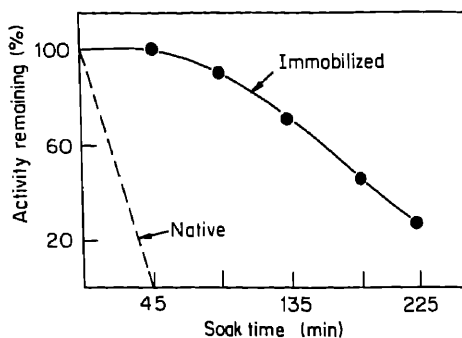
Figure 5. Soak time-activity relationship of immobilized trypsin and native trypsin in
guanidine HCL.

The present studies on the fractionation of sand by geochemical methods,
mination of potential reactive sites using $[2\text{-}^{14}C]$ malonic acid, denaturation s
using guanidine HCl and Scanning Electron Microscopy studies collec
indicated that the hydroxyl groups present on the surface of sand mig
responsible to a great extent for its binding with APTS and that trypsi
covalently coupled through APTS and glutaraldehyde.

Acknowledgement

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Microtubule associated proteins of goat brain

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Abstract. The microtubule associated proteins of goat brain were separated from tubulin on the basis of their thermostability and then fractionated by chromatography on Sepharose 4B column. Analysis of the fractions by SDS-polyacrylamide gel electrophoresis and assay of their tubulin-assembly-promoting activity indicate that this activity resides primarily in the tau-proteins (mol. wt. 55,000-70,000) and a class of even lower molecular weight (25,000-35,000) proteins. Electrophoresis of the microtubule associated protein fractions separated from tubulin by phosphocellulose chromatography are in agreement with the results obtained from fractionation on Sepharose 4B columns.

Introduction

Microtubule proteins isolated from various sources by cycles of temperature-dependent polymerization and depolymerization (Shelanski *et al.*, 1973) contain in addition to tubulin, several other associated proteins which copolymerize with tubulin. The nature and heterogeneity of these microtubule associated proteins vary in different tissues (Murphy and Borisy, 1975; Fellous *et al.*, 1977; Sloboda *et al.*, 1976; Cleveland *et al.*, 1977, 1979). Previous investigations (Fellous *et al.*, 1977; Sloboda *et al.*, 1976; Cleveland *et al.*, 1977) on the chracterization of these microtubule associated proteins and studies on their *in vitro* tubulin-assembly-promoting activity indicates that the major activity resides in two or three high molecular weight (250,000-350,000) proteins molecular weight and a set of four or five polypeptides of molecular weight 50,000-70,000 referred to as tau-proteins. More recently it has been demonstrated (Herzog and Weber, 1978) that both the high molecular weight proteins and the tau-proteins have independent tubulin-assembly-promoting activity but the morphology of the neurotubules formed in these two cases are different. The *in vivo* association of both tau and the high molecular weight proteins with microtubules in several types of cells has been demonstrated by using immunofluorescent antibodies (Conolloy *et al.*, 1977, 1978; Sherline and Schiavone, 1977). However, considerable doubt still remains as to whether all cells types contain the tau and high molecular weight proteins and the physiological role of these accessory proteins that copolymerize with tubulin (Cleveland *et al.*, 1979). In the present communication, we report the isolation of neurotubule proteins from goat brain, their fractionation by two independent procedures and identification of the tubulin-assembiy-promoting factors.

Materials and methods

Materials

Goat brains, from freshly slaughtered animals, were purchased from the local market. Sodium piperazine-N-N'-bis-2-ethane sulphonate, GTP type II, EGTA (ethylene glycol-bis β-amino ethyl ether-N, N'-tetra-acetic acid) and phosphocellulose were from Sigma Chemical Co., St. Louis, Missouri, USA. Sephadex G-25 and Sepharose 4B were from Pharmacia, Uppsala, Sweden. Acrylamide,(bis N, N'- methylene bisacrylamide) and Temed (N,N,N',N'-tetramethylene diamine) were from BioRad, Richmond, California, USA. All other chemicals were locally purchased and were of Analytical grade.

Preparation and fractionation of neurotubule proteins from goat brain

All operations except the temperature dependent polymerization of microtubule proteins were done at 4°C. Goat brains totally freed of meninges and blood vessels, were minced into small pieces and blended in a Waring blendor with piperazine ethane sulphonate buffer (0.1M buffer salt, 1mM $MgCl_2$ and 2mM EGTA, ph 6.45) using 1 ml buffer/g of tissue. The material was further homogenized in an all glass Dounce type homogenizer with a 'B' pestle and then centrifuged at 15000 g for 15 min. The supernatant was again centrifuged at 100000 g for 1 h. From the 100000 g supernatants, neurotubule proteins were purified according to the procedure of Shelanski *et al.* (1973) except that the temperature-dependent polymerization was carried out in the presence of 4 M glycerol at 37°C for 1 h and depolymerization at 0°C for 30 min. The final preparation, after two cycles of polymerization and depolymerization was clarified by centrifugation at 15000 g for 15 min and used for the isolation of microtubule associated proteins by boiling the purified neurotubule protein (Fellous *et al.*, 1977; Herzog and Weber, 1978). Phosphocellulose chromatography of neurotubule proteins were performed according to the procedure of Weingarten *et al.* (1975).

Miscellaneous procedures

SDS-polyacrylamide gel electrophoresis were done using a gradient of 4-15% acrylamide according to the procedure of Laemmli (1970); β-galactosidase, serum albumin, ovalbumin and α-chymotrypsinogen were used as molecular weight markers. Tubulin-assembly-promoting activity of the microtubule assembly proteins were measured by turbidimetric procedure at 350 nm using a thermostatically controlled (37°C) Beckman Model 24 spectrophotometer. The assembly of tubulin was temperature dependent and at a lower temperature, (0-4°C) the polymerized micro-tubules underwent depolymerization. The critical concentration of tubulin required for polymerization was 0.5 mg/ml. The time of equilibration at 37°C was 10-15 min. Protein was assayed by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

In view of the reports (Fellous *et al.*, 1977; Herzog and Weber, 1978) that, most or all of the microtubule associated proteins of rat or porcine brain can be separated from tubulin on the basis of their thermostability, initial isolation of the goat brain

microtubule assembly proteins was achieved by boiling purified neurotubule preparations, as described in 'Methods'. The Sepharose-4B elution pattern (figure 1) displayed a definite shoulder between two peaks. Four fractions, as indicated by bars, were collected for analysis by SDS-polyacrylamide gel electrophoresis and for assay of tubulin-assembly-promoting activity.
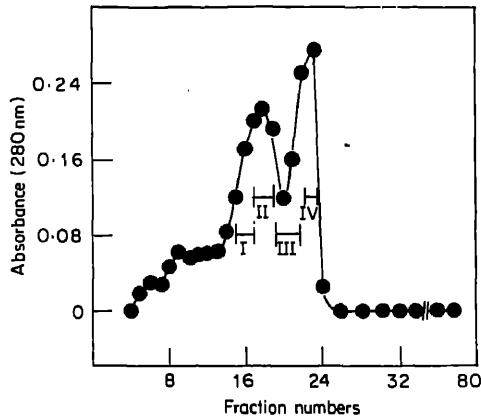
Figure 1. Elution pattern of goat microtubule assembly proteins from Sepharose 4B column. Total boiled factor (4 mg) was applied to the column and the proteins were eluted with piperazine ethane sulphonate buffer. Fractions indicated by bars (I to IV) were pooled separately.

The electrophoretic pattern of purified neurotubules show in addition to tubulin, about twenty bands with an extremely faint band in the region of high molecular weight proteins (Figure 2A, lane 2). The residue from the boiling step (lane 3) contains most of these proteins including the band in the region of the high molecular weight proteins. The tau proteins and some other proteins of low molecular weight (25,000 to 35,000) were the only major microtubule assembly proteins recovered (lane 4). The supernatant from the ammonium sulphate precipitation step did not show any distinct band (lane 5). Figure 2b Shows the gel pattern of the Sepharose 4B column fractions as well as the total boiled factor. While no protein band was detectable in the region of the high molecular weight proteins, several faint bands of 100,000-200,000 molecular weight were present in fractions I and II. These proteins may represent the subunits of the neuro-filaments which has recently been shown to copolymerize with tubulin (Runge *et al.*, 1981).

The tubulin-assembly-promoting activity of the total boiled factor was assayed in the presence and absence of 4M glycerol using phosphocellulose purified tubulin (phosphocellulose-tubulin). In the presence of glycerol, this tubulin alone underwent some polymerization after a lag of about 4 min (figure 3a). Addition of total boiled factor in increasing amounts promoted both the initial rate and the final level of polymerization with little or no lag. Identical concentrations of tubulin
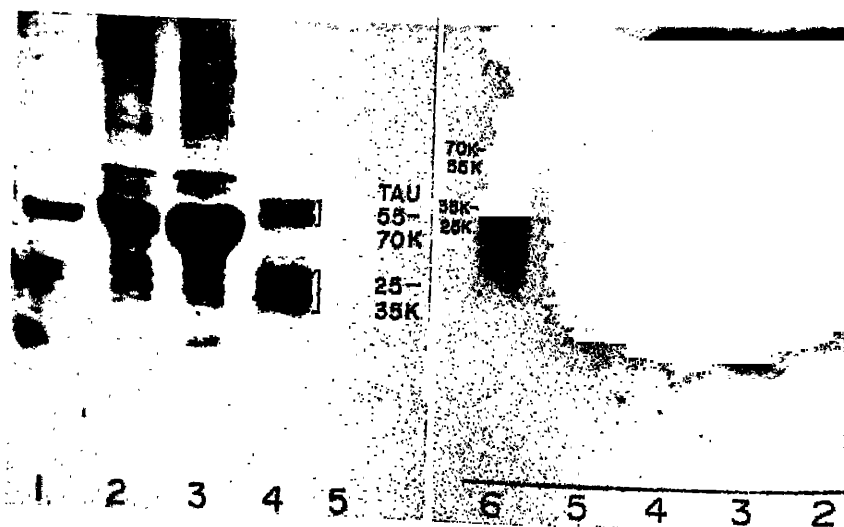
Figure 2. SDS-polyacrylamide gel electrophoretic patterns of goat neurotubule protein

(A) Lane 1—phosphocellulose tubulin (60 μg); lane 2—neurotubule protein purified by
cycles of polymerization; lane 3—precipitate after heat treatment of neurotubule protein;
4—total boiled factor recovered from the supernatant after heat treatment by precipita
with 50% ammonium sulphate; lane 5—supernatant from the ammonium sulphate precipita
step. Protein (60-90 μg) was applied in lanes 2-4. In lane 5, 50 μg supernatant was appli

(b) Lane 1—phosphocellulose-tubulin (75 μg); lane 2, 3, 4 and 5 represent fractions I to IV
45 μy) from the sepharose 4B column respectively; lane 6—total boiled factor (100 μg).
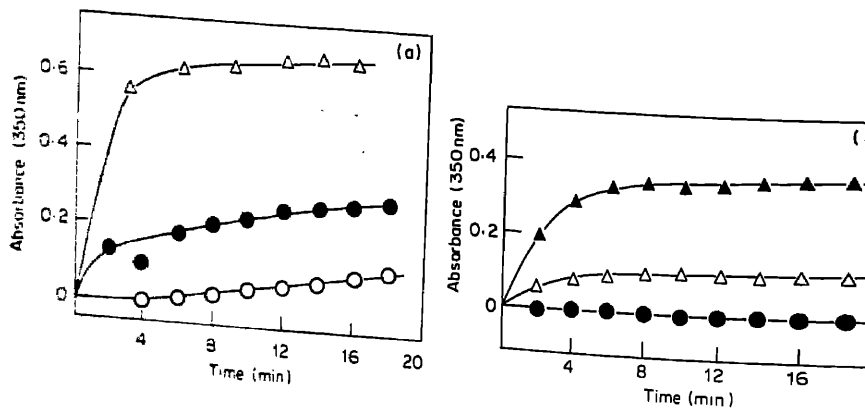
Figure 3. Tubulin-assembly-promoting activity of the total boiled factor in piperazine ethane
sulphonate buffer in the presence (a) or absence (b) of 4M glycerol. Phosphocellulose
tubulin concentration in all cases was 1.2 mg/ml. (a) phosphocellulose tublin alone (O);
tubulin: factor ratio, 10:1 (●); and 5:1 (△). (b) Phosphocellulosetubulin alone (●); tubulin:
factor ratio, 10:1 (△) and 5:1 (▲).

and the boiled factor were tested for polymerization in the absence of glycerol (Figure 3b); here, phosphocellulose tubulin alone was totally inactive and the activity of the boiled factor was about half of that observed in the presence of glycerol. In view of the marked effect of glycerol on the assembly of phosphocellu-lose-tubulin, all subsequent assays were performed in the presence of 4 M glycerol.

Figure 4 shows the relative tubulin-assembly-promoting activity of the total boiled factor, fraction II (peak I) and fraction IV (peak II) from the Sepharose column. These two fractions were assayed since the electrophoretic pattern of fraction II was very similar to that of fraction I and that of fraction III was almost identical to that of fraction IV (see Figure 2B). The results (figure 4) show that
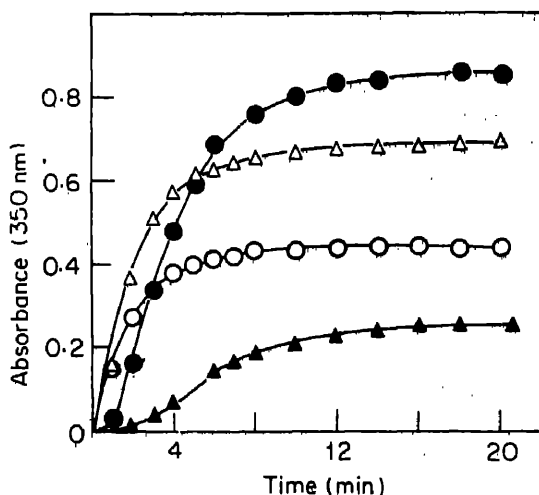
Figure 4. Tubulin-assembly-promoting activity of total boiled factor and the fractions eluted from the Sepharose 4B column in 4 *M* glycerol-piperazine ethane sulphonate buffer. ($\triangle$), phosphocellulose-tubulin (1.45 mg/ml) alone; ($\blacktriangle$), plus 0.1 mg of total boiled factor; (O), plus 0.1 mg fraction II from sepharose 4B column , ($\bullet$), plus 0.1 mg of fraction IV from sepharose 4B column.

both fraction II which is enriched with tau proteins, and fraction IV which is enriched with the low molecular weight proteins are individually more active than the unfractionated factor and that fraction IV is relatively more active than fraction II. The decreased activity of the unfractionated factor is likely to be due to the presence of inactive 100,000-200,000 molecular weight proteins which are seen primarily in fraction I, reduced in fraction II, and are totally absent in fraction IV (see figure 2B).

To examine if the 25000-35000 dalton proteins in the goat microtubule assembly proteins are artifacts of boiling or proteolytic degradation products of high molecular weight proteins, we have also fractionated the goat microtubule assembly proteins by chromatography on phosphocellulose columns (Weingarten

et al., 1978) using buffers containing 0.01 *M* phenylmethyl sulphonyl fluoride. Following the elution of all the tubulin from the columns, the microtubule assembly proteins were eluted in five steps using 0.1, 0.15, 0.2, 0.25 and 0.8 M NaCl. These fractions were concentrated by precipitation with 50% ammonium sulphate, dialysed against SDS-containing sample buffer (Laemmli, 1970) and subjected to electrophoresis in polyacrylamide gels. The electrophoretic pattern of these fractions (figure 5) displayed considerable similarity to those of the boiled factors, primarily showing two major classes of bands—the tau proteins and the low molecular weight proteins. In 0.15 M and 0.8 M NaCl (Lanes 2 and 5), a few proteins of molecular weight 100,000-150,000 appeared, but in none of the fractions the typical high molecular weight proteins were detectable. These later proteins however were consistently present in microtubule assembly proteins from embryonic or adult chick brain, fractionated by phosphocellulose chromatography under identical conditions.
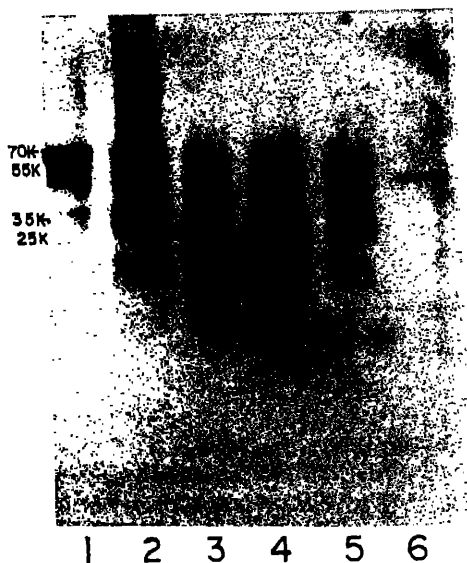
Figure 5. SDS-polyacrylamide gel electrophoretic pattern of the microtubule assembly protein fractions eluted from the phosphocellulose column by increasing concentrations of sodium chloride.

Lanes 1-5 represent fractions eluted with 0.1, 0.15, 0.2, 0.25 and 0.8*M* sodium chloride respectively, (50-70 µg proteins applied in each slot). Lane 6-phosphocellulose-tubulin (20 µg).

Discussion

In this communication, we show that microtubule assembly proteins from goat brain are characteristically different from those isolated from other sources in at least two aspects.

The major difference between goat and other vertebrate brain microtubule assembly proteins is with respect to the high molecular weight proteins—which are

either absent or present in quantities too small to detect in the goat brain. The *in vitro* tubulin-assembly-activity of both the tau proteins and the high molecular weight proteins have been well characterized in the case of neurotubules from calf (Sloboda *et al.*, 1976), porcine (Murphy and Borisy, 1975; Cleveland *et al.*, 1977), rat (Fellous *et al.*, 1977) and chick (Cleveland *et al.*, 1979) brain, and their association *in vivo* has also been well established (Conolly *et al.*, 1977, 1978, Sherline and Schiavone, 1977). High molecular weight proteins have been reported to be absent in the neuroblastoma (Nagle *et al.*, 1977) and other non-neural cell lines (Weatherbee *et al.*, 1978) and present in minute amounts in lamb brain (Maccionia and Seeds, 1978).

Fractionation of porcine brain microtubule assembly proteins by phosphocellulose chromatography showed (Herzog and Weber, 1978) that the high molecular weight proteins, free of tau, emerge from the column in buffers containing 0.1-0.2 *M* sodium chloride and the tau polypeptides appear at higher salt concentrations. In contrast, phosphocellulose chromatography of goat brain microtubule assembly proteins showed tau as the major protein in both 0.1 M and 0.15 M salt concentrations, no distinct band for high molecular weight protein was detected in these fractions. These findings and the polymerization activity data of the fractionated boiled factors (figure 4) indicate that the high molecular weight proteins, if present, represent a negligible fraction of the total goat microtubule assembly proteins and are unlikely to have any important physiological role.

The second characteristic feature in goat microtubule assembly proteins is the presence of the 25000-35000 dalton proteins in addition to the more commonly present tau proteins. These proteins are not degradation products or results of heat denaturation of the neurotubule proteins since they appeared in microtubule assembly proteins isolated by two different procedures and in buffers containing the protease inhibitor phenyl methyl sulphonyl fluoride. Comparison of the tubulin-assembly-promoting activity of these proteins with the tau proteins indicated that both classes of proteins are active (figure 4); however, quantitation of their relative activity require complete separation of these two classes of proteins which is yet to be achieved.

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Purification and properties of polyphenoloxidase of mango peel *(Mangifera indica)*

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Abstract. Polyphenoloxidase from mango *(Mangifera indica)* peel was purified to homogeneity by ammonium sulphate fractionation, chromatography on DEAE-Sephadex and gel filtration of Sephadex G-200. The enzyme had an apparent molecular weight of 136,000. Its pH and temperature optimum were 5.4 and 50°C, respectively. The enzyme possessed catecholase activity and was specific to *o*-dihydroxy phenols. The enzyme also exhibited peroxidase activity. Some non-oxidizable phenolic compounds inhibited the enzyme competitively. High inhibitory effects were also shown by some metal chelators and reducing agents. Mango peel polyphenol oxidase when immobilized onto DEAE Sephadex showed slightly higher K_m for catechol and lower pH and temperature optima.

Keywords. Mango *(Mangifera indica)* peel; polyphenoloxidase; peroxidase; immobilized enzyme.

Introduction

Polyphenoloxidase (EC 1.14.18.1) which plays a major role in the browning of plant tissues has been purified from a number of fruit tissues and their properties studied (Mayer and Harel, 1979; Jen and Flurkey, 1979; Jen *et al.,* 1980; Shin and Maier, 1980; Wissemann and Lee, 1980). The enzyme from many of the sources is shown to exist in multiple forms (Mayer and Harel, 1979). It is known to be a copper containing enzyme.

Polyphenol oxidase from *Mangifera indica* (mango peel, Badami variety) was partially purified and some of its properties namely, pH optimum, substrate specificity, and presence of copper in the enzyme were demonstrated in an earlier report (Venkiah and Patwardhan, 1977). This paper presents an improved method of purification of the enzyme from the same source leading to a homogeneous preparation thus enabling us to study many more of its properties in detail.

Attempts are being made to immobilize polyphenol oxidase for its possible use in instant tea manufacture (Prabha *et al.,* 1981). The present communication also reports the properties of the purified mango peel polyphenol oxidase immobilized onto DEAE-Sephadex as compared to the soluble enzyme.

Abbreviation used: PVP, polyvinyl pyrrolidone.

Materials and methods

Fully mature mango fruits (Badami variety) harvested from local gardens were allowed to ripen at room temperature. When they were edible ripe, the peel portion of the fruits was collected for enzyme isolation. DEAE-Sephadex A-50 and Sephadex G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden; standard proteins and polyphenols were from Sigma Chemical Co., St. Louis, Missouri, USA.

Enzyme isolation

Mango peel was blended in 1 mM potassium phosphate buffer, pH 7.4 containing 1% soluble polyvinyl pyrrolidone (PVP) and 0.1% ascorbic acid and acetone powder of this crude extract was prepared. Acetone powder was extracted for 1 h with about 10 volumes of 10 mM potassium phosphate buffer, pH 7.4 containing 1% insoluble PVP and 0.25% ascorbic acid. The viscous slurry thus obtained was clarified by treating with immobilized fungal pectinase for 2 h with intermittant stirring. Fungal (*Aspergillus carbonareus*) pectinase was immobilized by entrapping in polyacrylamide gel according to Chibata *et al.* (1976). The block gel containing entrapped pectinase was rubbed through a 40 mesh sieve and the small gel beads obtained were washed repeatedly with water to remove unbound pectinase. The polyphenoloxidase extract thus clarified was filtered through a cheese cloth and centrifuged at 10,000 *g* for 10 min to get a clear solution.

Enzyme purification

All purification steps were carried out at 4°C. The enzyme was precipated from the crude extract at 70-80% ammonium sulphate saturation and suspended in a minimum volume of 0.05M phosphate buffer, pH 7.4 and dialyzed against 0.01M phosphate buffer (3L) overnight.

The above enzyme was placed on a DEAE-Sephadex column (3.5×13 cm). Potassium phosphate buffer, pH 7.4, 0.025M, 0.05M, 0.075M and 0.1M (flow rate: 12 ml/h) 100 ml were passed through the column. The enzyme activity was detected in the fraction eluted with 0.1M phosphate buffer. The eluate from DEAE sephadex column was concentrated by lyophilization to about 2 ml and further purified by gel filtration.

Gel filtration of the enzyme was carried out on a Sephadex G-200 column (1.4×150 cm; flow rate: 5ml/h) with 0.1M potassium phosphate buffer, pH 7.4. The enzyme fractions were pooled and used in the study.

For molecular weight determination, the enzyme was rechromatographed (Whitaker, 1963) on the same column previously calibrated with blue dextran and other standard proteins. The proteins were monitored by their absorbance at 280 nm. The enzyme was monitored by assaying for activity.

Enzyme assay

Polyphenol oxidase activity was measured spectrophotometrically by the method described by Wong *et al.* (1971) using 10 mM catechol as substrate. One unit of the enzyme activity was taken as increase in absorbance at 420 nm of 0.01 per min per mg protein. In the case of immobilized preparation, about 2g of the material

(drained) was incubated with 10 ml of 20 mM catechol for 60 min at 25°C. The absorbance of the supernatant solution was measured at 420 nm. The reading was recorded every 15 min. Here, the activity unit was taken as increase in absorbance at 420 nm of 0.01 per 30 min per mg protein. To determine the enzyme activity in the presence of inhibitors, the enzyme was preincubated for about 15 min with these compounds.

Peroxidase activity was measured by the method described by Vance and Sherwood (1976). Protein was estimated according to Lowry *et al.* (1951).

Electrophoresis

Disc gel electrophoresis of the enzyme preparations (sample containing ca. 100 μg protein) was carried out on 7.5% polyacrylamide gels using Tris-glycine buffer, pH 8.3 according to Davis (1964). The gels were stained with 5% amidoblack solution for 30 min and destained with 7% acetic acid to develop protein bands. The polyphenoloxidase bands were detected by dipping the gels in 10 mM substrate solutions containing 0.05% *p*-phenylenediamine (Montgomery and Sgarbieri, 1975). Peroxidase bands were developed with hydrogen peroxide and *o*-dianisidine (Shannon *et al.*, 1966).

Immobilization by adsorption of polyphenol oxidase on to DEAE Sephadex

Adsorption of the enzyme on DEAE-Sephadex A-50 was achieved by suspending about 20 ml of the enzyme solution with 5 g (wet weight) of pre-conditioned support at pH 7.4 using 0.1M phosphate buffer with constant stirring for 2 h as described by Park and Toma (1975). This was then washed free of unbound enzyme. Quantity of protein held on the support was calculated by subtracting the protein present in the combined washings of the immobilized support from the protein taken for immobilization.

Results

Table 1 gives the results of purification of polyphenol oxidase. More than 90% of the enzyme protein was precipitated at 70-85% ammonium sulphate saturation.

Table 1. Purification of mango peel polyphenoloxidase.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purification	Recovery (%)
Extract from acetone powder	1738	1297	1.34	1	100
Ammonium sulphate (70-85% saturation)	1623	42.5	38.2	29	93
DEAE-Sephadex A-50	689	8.96	76.9	57	40
Sephadex G-200	187	1.40	133.6	100	11

The enzyme fraction after ion exchange chromatography with DEAE Sephadex and gel filtration on Sephadex G-200 was nearly 100-fold purer. Polyacrylamide gel electrophoresis of this fraction revealed a single protein band. This enzyme protein corresponded to the activity band when stained for activity (figure 1) and
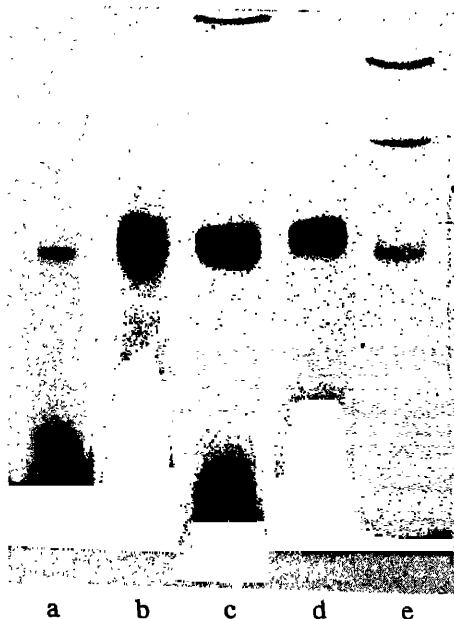
Figure 1. Polyacrylamide gel electrophoretic pattern of different fractions obtained during purification of mango peel polyphenoloxidase.

(a) 70-85% ammonium sulphate fraction; (b) DEAE Sephadex fraction; (c) Sephadex G-200 fraction.

(a), (b) and (c) are stained for polyphenol oxidase activity; (d) same as (c) but stained for proteins; (e) same as (c) but stained for peroxidase activity.

this corresponded with one of the activity bands in the crude extract. The same protein band also possessed peroxidase activity when stained for peroxidase. The four isoenzymes of polyphenoloxidase present in the crude extracts exhibited different substrate specificities, as revealed by the differential appearance of activity bands upon incubation with catechol, dopa or dopamine, gallic acid, chlorogenic acid and caffeic acid.

The molecular weight of the purified polyphenol oxidase isoenzyme was found to be 136,000 by gel filtration (figure 2). The purified enzyme had optimal activity at pH 5.4 and at 50°C. Figure 3 represents temperature stability curve of the purified polyphenoloxidase. Significant loss in activity was noticed at temperature 70°C and above.
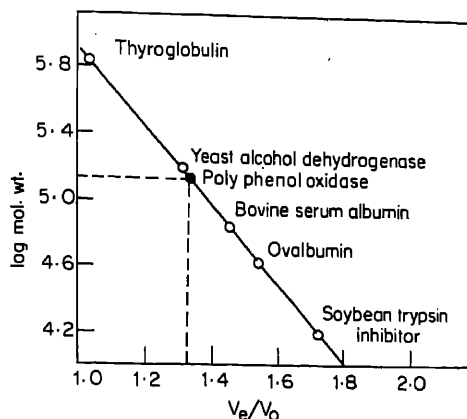
Figure 2. Molecular weight determination of mango peel polyphenoloxidase by gel filtration on Sephadex G-200.
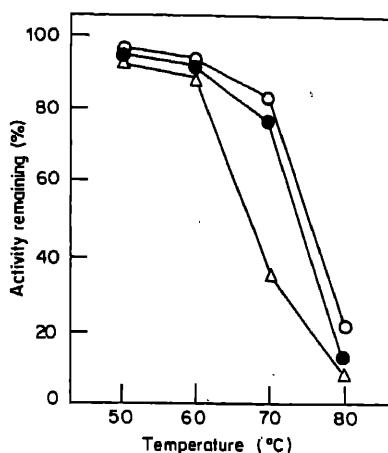
Figure 3. Temperature stability curve for mango peel polyphenoloxidase.
15 min, (O); 30 min, (●); and 60 min, (Δ)

The substrate specificity of the purified enzyme is shown in table 2. Catechin and epicatechin had an oxidation rate which was about 75% higher than that of catechol, whereas other substrates exhibited nearly 50% lower activity than catechol. The enzyme was inactive towards hydroquinone, p-coumaric acid and tyrosine. K_m values of the enzyme for various phenolics derived from Lineweaver-Burk plots were 3.49 mM, catechol; 1.9 mM, catechin; 2.0 mM, epicatechnin; 8.9 mM, gallic acid; 6.9 mM, dopamine and 12.4 mM, caffeic acid.

Inhibition of polyphenoloxidase by various classes of inhibitors is shown in table 3. Some of the non-oxidizable phenolics, viz., hydroquinone, pyrogallol and α-naphthol inhibited the enzyme activity by about 80% at 1 mM concentration. The inhibition of the enzyme by these phenolics except p-coumaric acid was competitive

Table 2. Specificity of the purified mango peel polyphenol oxidase.

Phenolic compound	Enzyme activity*	K_m value
Catechol	100	3.49
Catechin	178	1.90
Epicatechin	172	2.00
Dopamine	59	6.90
Gallic acid	54	8.90
Caffeic acid	48	12.40
Chlorogenic acid	45	—
Protocatechuic acid	42	—
Hydroquinone	0	—
p-Coumaric acid	0	—
Tyrosine	0	—

* Phenolics were added at 10mM concentration.
  Activities are expressed relative to catechol, which is taken as 100.
  — Not determined.

Table 3. Inhibition of mango peel polyphenol oxidase by different classes of inhibitors

Inhibitor	Per cent inhibition at (mM) 1.0 0.1	Inhibitor	Per cent inhibition at (mM)				
			1.0	0.1	0.01	0.001	0.0001
Phenolic compounds:		*Metal chelators:*					
Hydroquinone	85 65	3,4-Dichlorophenyl	100	100	100	85	12
Pyrogallol	78 65	serine					
a-Naphthol	80 46	Sodium azide	98	92	66	8	—
p-Coumaric acid	32 20	Sodium diethyl-	100	95	40	—	—
Ferulic acid	40 0	dithiocarbamate					
Other phenolics*	0 0	Phenylthiourea	96	76	26	—	—
		EDTA	0	0	0	—	—
Miscellaneous compounds:		*Reducing agents:*					
Calcium chloride	14 —	Glutathione	100	100	100	85	58
PVP	11 —	Cysteine.	100	100	75	—	—
Borate	0 —	Potassium meta-	100	100	20	—	—
		bisulpite					
		Ascorbic acid	100	100	42	—	—

* Compounds like benzoic acid, salicylic acid, resorcinol, cinnamic acid, p-chlorophenol, m-,
  o- and p-cresols and diacetyl.
  — Not determined.

as evidenced by the Lineweaver-Burk plots (figure 4). The K_m values of the enzyme for catechol in presence of hydroquinone, pyrogallol, α-naphthol and *p*-coumaric acid increased from 3.49 to 20.16, 17.64, 15.12 and 5.7 mM respectively (figure 4). As indicated in table 3, other phenolics did not inhibit the enzyme even at 1 mM concentration.
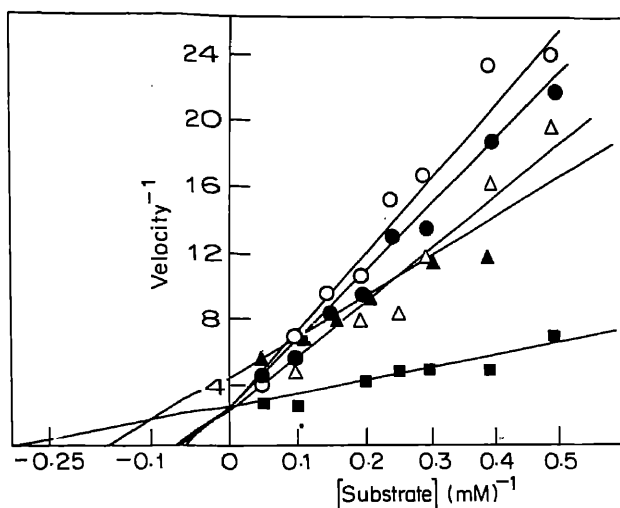
Figure 4. Lineweaver-Burk plots of mango peel polyphenoloxidase for catechol in presence of phenolic inhibitors (10 mM).

Hydroquinone (O); pyrogallol (●) ; α-naphthol (Δ); p-coumaric acid (▲); control (■).

Metal chelating compounds, namely 3,4-dichlorophenyl serine, sodium azide, sodium diethyldithiocarbamate and phenylthiourea caused more than 75% inhibition at 0.1 mM concentration. 3,4-Dichlorophenyl serine was highly inhibitive even at 1 mM level. EDTA did not show an inhibition. The reducing agents-glutathione, cysteine, potassium metabisulphite and ascorbic acid completely inhibited the enzyme activity at 0.1 mM level, glutathione being the most potent. Other compounds like calcium chloride, borate and PVP at 1 mM level showed very little effect on mango peel polyphenoloxidase.

The effect of some of the selected inhibitors of polyphenoloxidase on peroxidase activity of the same enzyme protein is given in table 4. *p*-Coumaric acid and ferulic acid at 1 mM level showed a high activation of peroxidase activity while inhibiting the polyphenoloxidase activity of the protein. 3,4-Dichlorophenyl serine (copper chelator and a potent inhibitor of polyphenol oxidase) had no effect on peroxidase activity. On the other hand, 1,10-phenanthroline hydrate (a specific iron chelator) brought about total inhibition of peroxidase activity even at 10 μM level and this metal chelator had no effect on polyphenoloxidase activity.

Table 4. Effect of some inhibitors of polyphenol oxidase on peroxidase activity of the purified enzyme proteins.

Compound	Peroxidase activity				Polyphenol oxidase acti·		
	1 mM	0.5 mM	0.1 mM	0.01 mM	1 mM	0.001 ml
p-Coumaric acid	(+) 162	(+) 36	(+) 8	0	·(−) 32	0
Ferulic acid	(+)1552	(+)1404	(+)900	(+) 89	(−) 40	0
1,10-Phenanthro-line hydrate	(−) 100	(−) 100	(−)100	(−)100	0	0
3,4-dichloro-phenyl serine	0	0	0	0	(−)100	(−)84

Values are expressed as per cent increase or decrease over the control.
(+): activation; (−): inhibition; 0: no effect.

The enzyme exhibited good storage stability. The enzyme stored in pH 7 buffer at room temperature for 72 h or at 5°C for 2 months retained 95% of th original activity. But at pH 5.5, the enzyme lost about 50% of the activity und similar conditions. Freeze drying, freezing and thawing did not affect the activi of the enzyme. Acetone powders which were stored at −15°C retained 90% of th activity upto 4 months. A storage period of 6, 8 and 10 months decreased th activity to 60, 55 and 30% respectively. The isoenzyme pattern of mango pe polyphenoloxidase from acetone powers stored for more than 8 months show(diffused bands of the enzyme with a poor resolution.

Properties of the immobilized polyphenoloxidase

The purified enzyme immobilized onto DEAE Sephadex showed linear activi between 10 and 70 min, as against 0-4 min for the soluble enzyme. The insolubl enzyme exhibited an optimum pH of 5.0 and an optimum temperature of 44°C The immobilized enzyme exposed for 60 min to 50, 60, 70 and 80°C retained 90, 8(22 and 0% activity respectively. The K_m of the bound enzyme for catechol was 7. mM which was 3.49 for the soluble enzyme. The immobilized enzyme showe higher activity towards catechin and epicatechin as compared to catechol (3 an 2.9-fold respectively) and showed much lower activity towards dopamine, galli acid, chlorogenic acid and caffeic acid than catechol (0.4, 0.42, 0.4 and 0.38 fol respectivley). The specific activity of the native enzyme decreased upon immob lization by about 18%. The immobilized mango peel polyphenoloxidase retaine more than 90% of its activity even at the end of six months storage at 4°C.

Discussion

In this study, mango peel polyphenoloxidase has been obtained in an electrophore tically homogeneous form. This purified enzyme protein corresponds t(isoenzyme-2 of the four isoenzyme bands originally present in the crude extract

The only report on the existence of isoenzyme forms for mango peel polyphenoloxidase has been that of Park *et al.* (1980) who reported two isoenzymes reacting with catechol in Haden variety of mango. The four isoenzymes of mango peel noticed in our study exhibited differences in their reactivity towards various substrates, of which the isoenzyme-2, was reactive to all *o*-dihydroxyphenols and was the most prominent one.

Venkiah and Patwardhan (1977) purified mango peel (Badami variety) polyphenoloxidase to 50-fold with 4% recovery by ion-exchange chromatography and demonstrated the presence of copper, the substrate specificity and the pH optimum for the partially purified preparation. The polyphenoloxidase enzyme from mango peel has been purified to homogenity with about 11% recovery in the present investigation.

The purified mango peel enzyme has peroxidase activity, similar to the polyphenoloxidase from other sources (Shannon *et al.,* 1966; Bayse and Morrison, 1971; Hideakishinishi, 1975). 1, 10-Phenanthroline hydrate, a specific iron chelator inhibits peroxidase activity of the mango peel enzyme, but not polyphenoloxidase activity. Similarly, 3, 4-dichlorophenyl serine, a specific copper chelator inhibited polyphenoloxidase activity but not peroxidase activity. Thus, it could be inferred that the purified enzyme from mango peel is a metalloprotein containing both copper and iron. Further, copper is not involved in peroxidase activity of the protein and iron is not involved in polyphenoloxidase activity of the enzyme protein.

Immobilization of mango peel polyphenol oxidase by adsorption onto DEAE Sephadex has resulted in slight shifts in pH and temperature optimum towards the lower side. The K_m for catechol has increased from 3.49 to 7.2 mM, associated with fall in specific activity. Similar loss in specific activity upon immobilization has been reported for aminoacylase adsorbed onto DEAE-Sephadex (Tosa *et al.,* 1966). Loss in enzyme activity upon immobilization has been attributed to change in enzyme conformation imposing rigidity and hindered accessibility of the enzyme to substrate (Zaborsky, 1974). Immobilization of mango peel polyphenoloxidase did not bring about drastic changes in specificity of the enzyme towards phenolic substrates. Immobilized preparation of the enzyme suspended in buffer and kept at 4°C has shown good storage stability for 6 months. Generally, immobilization brings about modifications in the properties of the enzyme as has been reviewed by Zaborsky (1974).

During extraction of ripe mango peel with buffer, a lot of pectin was co-extracted resulting in a highly viscous non-filterable mass, which prevented a clean separation of the aqueous phase containing the enzyme. To overcome this problem, pectinase was made use of in our work. Immobilized pectinase (entrapped in polyacrylamide gel) for clarifying the mango peel extract was most advantageous and therefore was adopted. Immobilized pectinase was preferred to soluble enzyme as the former could be removed easily after use so that additional protein contamination is avoided.

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Isolation and characterization: of α -santonin assimilating Pseudomonad

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Abstract.   *Pseudomonas cichorii* strain *S*, isolated by soil enrichment technique, utilized santonin as the sole source of carbon, forming chromatographically destinguishable transformation products. One of the intermediary transformation products was identified as 1,2-dihydro α -santonin.

Keywords.   α -santonin; microbial transformation; *Pseudomonas cichorii*; 1, 2-dihydro α -santonin.

Introduction

α-Santonin (santonin) (figure 1, I), a pharmaceutically important sesquiterpenoid, has recently assumed significance as a potential parent compound for antitumour drugs (Fujimoto *et al.*, 1979). Its chemical conversions are being worked out extensively. The microbial transformation, though viewed as a useful tool for obtaining newer derivatives, has not yet yielded successful results, as santonin itself is not easily amenable to microbial attack (Fujimoto *et al.*, 1979). The transforma-
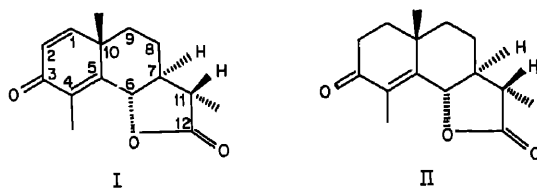
Figure 1.   Structure of α-santonin (1) and its transformation products, 1-2-dihydro α -santonin (II).

tion products which are detected by using mixed cultures are in small quantities and hence, pose difficulty during purification (Hikino *et al.*, 1970). Resistance of santonin to microbial conversion led Fujimoto *et al.* (1979) to use chemical analogues for microbial transformation. We report our findings on the enrichment, isolation and chracterization of a single bacterial strain, capable of utilizing santonin as the sole source of carbon.

Materials and methods

Media

Santonin medium used for enrichment, isolation and other growth stu⟨ consisted of mineral salts medium (Mahtani and Mavinkurve, 1979) suppleme⟨ with 0.5% (w /v) α-santonin I.P. Santonin agar was prepared by solidifying above medium with 2% bacto-agar (Difco). Media were sterilized at $121°C$ f⟨ min.

Enrichment, isolation and characterization

Garden soil was enriched by regular additions of santonin (Lonsane *et al.,* 1⟨ This soil was then used as inoculum in santonin medium. The enriched cul⟨ thus obtained were inoculated on nutrient agar or santonin agar for isolation purification of the organisms using santonin as the carbon source.

The isolates were characterized morphologically and biochemically (Socie⟨ American Bacteriologists, 1957). The G+C content of the DNA was determ⟨ as reported by Mandel (1966) and cultures were further identified (Buchanan Gibbons, 1974; Stanier *et al.,* 1966 and Sands *et al.,* 1970).

Utilization and transformation of santonin

The isolate capable of utilizing santonin was inoculated into the santonin me⟨ and incubated on a rotary shaker at room temperature (28-32°C). The growth followed turbidimetrically using a Klett Summerson Photoelectric Colorim⟨ One ml aliquots of the culture broth were withdrawn at intervals and extracted t with 0.5 ml chloroform. Individual chloroform extracts were analyzed by thin l chromatography and for ultraviolet absorbance profiles.

Characterization of one of the transformation products

The culture was grown in 3 litres of santonin (0.3%) medium for 24 h. The br⟨ after removing the cells by centrifugation, was extracted with chloroform. extract was concentrated by vacuum evaporation and the products were separ⟨ by multiple run (×4) preparative thin layer chromatography using petrol⟨ ether 40-60°C and ethyl acetate (9:1) solvent systems. The separated bands ⟨ eluted with ethyl acetate. The chromatographically pure product obtained characterized spectroscopically.

Results

Isolation and identification of santonin utilizing culture

The soil enrichment technique yielded a mixed bacterial culture capabl⟨ utilizing santonin as the sole source of carbon, which could be maintaine⟨ regular transfers in the santonin medium. The enriched culture on platin⟨ nutrient agar, gave consistently two isolates, identified subsequentl⟨ *Pseudomonas putida* and *P. solanacearum* which either individually or ⟨ together failed to grow in santonin medium. The mixed culture plated on sant⟨ agar gave rise to minute colonies of slow growing organism. The purified iso⟨

showed a growth pattern similar to that of parent mixed culture in santonin medium. The isolate consisted of strictly aerobic, gram negative, non-sporulating 0.9 to 1.26 μm long coccobacilli, arranged singly or in pairs and actively motile with polar multitrichous flagella. The characteristics of the isolate including G+C content (table 1) confirm its identity as *Pseudomonas cichorii* (Buchanan and Gibbons, 1974; Sands *et al.*, 1970) and is designated as strain *S*.

Table 1. Physiological characteristics of the strain S.

Growth at 37°C	—
Production of pigments	
Fluorescence	+
Pyocyanin and carotenoide	—
Nitrate reduction	—
Arginine dihydrolase	—
Catalase	+
Oxidase	+
Urease	—
Indole production	—
H₂S production	—
Hydrolysis of	
Poly-β-hydroxy butyrate	—
Gelatin	—
Starch	—
G+C content (moles %)	58.8
Carbon sources for growth	
Glucose	+
Lactate	—
Benzoate	+
β-hydroxy butyrate	—
Arginine	—
Cleavage of diphenols	*Ortho*

Santonin utilization by Pseudomonas cichorii strain S

The growth of *Pseudomonas cichorii* strain *S* on santonin agar showed a clear halo around the colonies due to dissolution of santonin crystals (figure 2). Each of the chloroform extracts of the culture broth, withdrawn at intervals and scanned individually, showed a single λ max at 242 nm, the absorbance being decreased as a function of increase in bacterial growth in santonin medium.

Transformation pattern of santonin during the growth of Pseudomonas cichorii strain S

The spots visualized on thin layer chromatograms either by iodine vapours or by spraying with 50% sulphuric acid or under ultraviolet light after spraying with rhodamine B gave identical results indicating sequential formation of the intermediate product during the growth of isolate designated as *L, M, N, O,* and *P* (figure 3). None of these products however, could be detected when the organism was grown on acetate, glucose or benzoate.
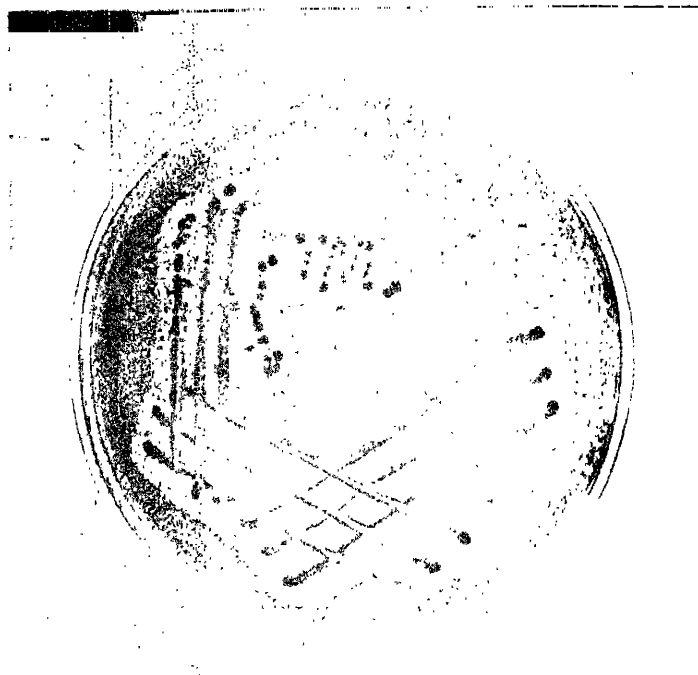
Figure 2. Colonies of *P. cichorii* strain *S* on santonin agar.
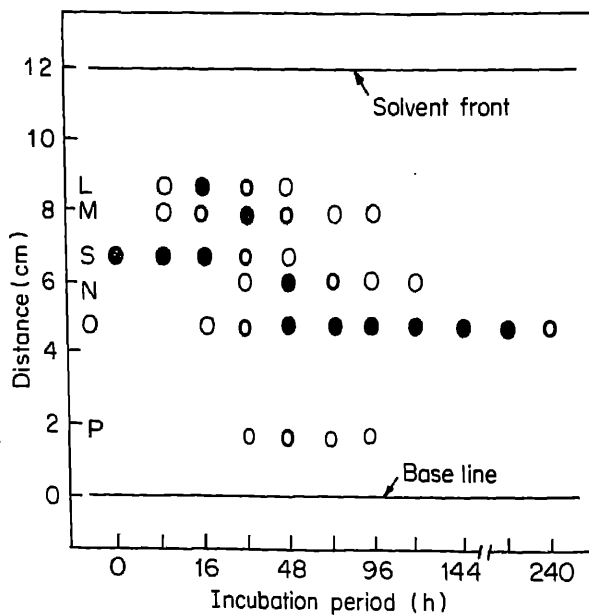

Figure 3. Thin layer chromatograms of the chloroform extracts of the culture medium, during the growth of *P. cichorii* strain *S* in santonin medium. Supports: Silica gel C; Solvent system: Benzene and ethyl acetate (1:1); Visualizing agent: Iodine vapours; S: Santonin; *L, M, N, O, P;* Transformation products; O, **O**, ●: intensities of the spots in increasing order.

Microbially transformed product of α-santonin

The transformation product M which appeared as a single substance initially, resolved into two components during preparative multiple run thin layer chromatography. One of the purified components M_1, analysed spectroscopically has the molecular formula $C_{15}H_{20}O_3$ (M+248). Its infrared and nuclear magnetic resonance spectra indicated the absence of C_1-C_2 unsaturation with the remaining structural features intact. Spectral comparison and co-migration on chromatogram with authentic 1,2 dihydro santonin prepared by partial hydrogenation of santonin over 10% palladised charcoal established the identity of M_1 with 1,2-dihydro santonin (figure 1, II).

The santonin utilizing Pseudomonad grows luxuriently on the authentic derivative (figure 1, II) as sole source of carbon forming similar intermediates as those from α-santonin. The santonin grown washed cells of *P. cichorii* strain *S* showed slightly higher oxygen uptake with 1,2-dihydrosantonin (6.08 ppm/h/mg dry wt) than that with santonin (5.03 ppm/h/mg).

Discussion

The soil enrichment technique yielded the santonin utilizing mixed bacterial culture, in which two strains of *Pseudomonas* proliferated consistently, apparently by utilizing some of the products formed from santonin by strain *S*. Synergism of this kind has been reported earlier during metabolism of quarternary ammonium compounds (Dean-Raymonds and Alexander, 1977). The elusiveness of strain *S* during initial isolation from the mixed enriched culture may be attributed to its slow growth, forming barely visible colonies on nutrient agar after 48 h; a characteristic uncommon for *Pseudomonas* but a distinctive feature of *Pseudomonas cichorii* (Buchanan and Gibbons, 1974). The only inconsistency of strain *S* from the other reports for *P. cichorii* was with respect to it lack of hypersensitivity towards *Nicotiana tobacum* (Klement, 1963), and plant of *Cichorium* sp. Variations of this type are known to occur in cultures continuously maintained in the laboratory (Buddenhagen and Kelman, 1964).

The dissolution of santonin crystals in agar around the growing colonies (figure 2) implies the release of certain extracellular factors for solubilization or transport of santonin, perhaps similar to those described for *n*-alkanes (Hisatsuka *et al.,* 1977).

The reduction of santonin concentration during growth is unequivocally established from the disappearance of santonin (figure 3) and the steep decrease in absorbance at 242 nm (λ max of α-santonin) of chloroform extracts of culture broth. Some of the transformation products appear to retain the conjugated carboxyl grouping intact which evidently contribute to the residual absorbance at 242 nm even after the disappearance of santonin after 72 h.

Spectral features of the transofmration product M_1, and growth and oxygen uptake by *Pseudomonas cichorii* strain *S* with authentic 1,2-dihydrosantonin, confirm the latter to be one of the initial metabolites in the pathway of utilization of santonin by the culture. It is of interest to note that the same product was also reported as a cometabolite of santonin, formed by *Cunnhinghamella blakesleeana*

and *Streptomyces aureofaciens* (Hikino *et al.*, 1970).   The reduction of the d
bond at 1-2 position appears to be an initial step of microbial attack on santo
well as its analogues (Fujimoto, *et al.*, 1979).

The sequential appearance followed by disappearance of the other tra
metabolites during the growth indicate the potential usefulness of the syst
elucidating the pathway of santonin catabolism.

To our knowledge, the utilization of santonin as a sole source of carbo
energy by a single organism is not yet reported.   The elucidation of the stru
of the transformation products is expected to be rewarding not only i
biodegradation studies but may also prove fruitful in emergence of some
pharamaceutically important products.

Acknowledgements

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Control of sporulation in the filamentous cyanobacterium *Anabaena torulosa*

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Abstract. In the cyanobacterium *Anabaena torulosa*, sporulation occurred even during the logarithmic growth phase. Sporulation was initiated by differentiation of the vegetative cell on one side, adjoining the heterocyst followed by differentiation of the vegetative cell on the other side. Subsequently, spores were differentiated alternately on either side to form spore strings. The sequence of sporulation supports the previous notion that a gradient of spore maturation exists in cyanobacteria and also indicates that the gradient is manifested unequally on either side of heterocysts. Sporulation was absent or negligible in a minerally enriched medium but ocurred readily in a minimal medium. The extent of sporulation was inversely related to phosphate concentration. Sporulation was enhanced at higher temperature. Incandescent light, but not fluorescent light, greatly stimulated sporulation suggesting possible involvement of red light in spore differentiation. Addition of filtrate, from 5 to 8 day old cultures, to freshly inoculated *A. torulosa* greatly enhanced sporulation indicating the influence of extracellular products in spore formation.

Keywords. *Anabaena torulosa;* akinete sporulation; cyanobacterium; blue-green alga.

Introduction

Many filamentous cyanobacteria are characterized by the presence of a third cell type called spore or akinete in addition to the vegetative cell and the heterocyst. The three cell types occur in patterned sequence which differ from species to species. For instance, in *Anabaena cylindrica,* spores are differentiated adjacent to heterocysts (Wolk, 1965), whereas in *Anabaena doliolum* (Tyagi, 1974) and *Nostoc* PCC 7524 (Sutherland *et al.,* 1979) spores start to differentiate midway between two heterocysts. Heterocysts have been implicated in the regulation of spore formation and a gradient of spore maturation has been proposed (Wolk, 1965; Tyagi, 1974; Simon, 1977). However, it has been reported (Sinha and Kumar, 1973) that *A. doliolum* is able to sporulate without first forming heterocysts and in *Nostoc linckia* heterocysts seem to inhibit sporulation (Singh *et al.,* 1972). The normal pattern of spore formation in *A. cylindrica* is drastically altered by provision of the arginine analogue canavanine and spores develop randomly in the filament (Nichols *et al.,* 1980).

The conditions of culture and physiological factors favouring spore formation have not been adequately documented, although some of these features are known

(Wolk, 1975; Nichols and Carr, 1978). Phosphate deficiency stimulates sporulation in *Cylindrospermum* (Glade, 1914; Fisher and Wolk, 1976), *Anabaena cylindrica* (Wolk, 1965) and *Nodularia spumigena* (Pandey and Talpasayi, 1980). High light intensity, high concentration of phosphate (Wolk, 1965), potassium nitrate and ammonium chloride (Tyagi, 1974) have been shown to inhibit spore formation in cyanobacteria whereas certain amino acids, calcium glucuronate and hydrogen enhance sporulation in *C. licheniforme* (Hirosawa and Wolk, 1979a). The presence of an extracellular positive effector which stimulates sporulation was demonstrated in *Cylindrospermum licheniforme* (Fisher and Wolk,1976) and a low molecular weight (M_r 151) active substance which appeared to be the major single effector has since been purified to homogeneity from this organism (Hirosawa and Wolk, 1979b). In this paper, the factors favouring sporulation in *Anabaena torulosa* are described. Unlike other cyanobacteria examined so far, where sporulation occurs in stationary phase cultures, *A. torulosa* initiates sporulation even during logarithmic growth. Sporulation could therefore be studied independently of the effects of decreased growth rates.

Materials and methods

Organism

Anabaena torulosa Lagerh. (see Desikachary, 1959) isolated in axenic culture in this laboratory (Fernandes, 1978) was the experimental material.

Culture conditions

Stock cultures were maintained on agar slants (1%, w/v Difco agar) enriched with two fold diluted cyanophycean medium (David and Thomas, 1979). Cultures for experimental purpose were grown as required in the diluted cyanophycean medium, or in Glade's medium (Glade 1914) which has the following composition (mg/litre): $Ca(NO_3)_2.4H_2O$, 1000; $MgSO_4.7H_2O$, 200; K_2HPO_4, 200; $FeSO_4.7H_2O$, 50. Other conditions for routine growth of culutres were as described before (Thomas 1972). Erlenmeyer flasks (500 ml or 1 litre) were used and 4000 lux light intensity was provided by a bank of fluorescent lamps. Temperature was $28 \pm 1^\circ C$. The cultures, 5 cm deep, were aerated at the rate of 2 litre per min.

Cell counts and growth determinations

Counts of vegetative cells, heterocysts and spores in the algal filaments were made as described earlier (Thomas and David, 1971). Those cells which were having thick walls and were at least twice as long and distinctly wider than the average vegetative cell (Simon, 1977) were considered as spores. Total protein content remains unchanged in sporulating cyanobacteria, whereas dry weight and chlorophyll content increase in a linear manner (Simon 1977). The problem of dehydrated mucilage rapidly absorbing moisture results in erratic dry weight measurements. Growth was therefore assessed in this study by determining chlorophyll content (Mackinney, 1941).

Sporulation in liquid and solid media and the effect of temperature

One ml suspension of a six-day old aerated culture of *A. torulosa* was inoculated into

Erlenmeyer (150 ml) flasks containing either the diluted cyanophycean medium (liquid) or containing 1% (w/v) agar (solid) prepared in diluted cyanophycean medium. Sets of flasks were incubated at 29, 32 and 37°C as still cultures. Temperature was adjusted by using a water bath. Incandescent light (1000 lux) was provided by tungsten filament lamps. The effect of temperature was also examined in aerated cultures grown in diluted (1:3) Glade's medium at 32 and 40°C in the presence of incandescent light (3000 lux).

Effect of light source and intensity of sporulation

A. torulosa was inoculated in diluted (1:3) Glade's medium. Sets of flasks were incubated in fluorescent light (3000 lux) or incandescent light (3000 lux) at 29°C. Effect of light intensity on sporulation was examined using incandescent light at 1000, 3000 and 5000 lux.

Effect of phosphate on sporulation

Six-day old cultures grown in cyanophycean medium, centrifuged, washed and resuspended in sterile distilled water, served as inoculum. Sporulation was then examined during growth in diluted (1:3) Glade's medium containing 0.3, 0.7, 1.0 and 1.4 mM phosphate. The culture flasks were incubated at an intensity of 3000 lux incandescent light.

Stimulation of sporulation by culture filtrate

A. torulosa was grown in diluted (1:3) Glade's medium 1 litre conical flasks at 40°C with incandescent light (3000 lux). After 8 days, the cultures were centrifuged and the supernatant was passed through Millipore filter (0.45 µm). The culture filtrate so obtained was mixed with fresh Glade's medium in various proportions. Five day old *A. torulosa* grown in cyanophycean medium was harvested, washed in distilled water and then inoculated into the media containing the various proportions of the culture filtrate.

Sequence of sporulation

The time course and sequence in which spores appear on either side adjacent to heterocysts were examined in cultures grown in Glade's diluted medium with incandescent light (3000 lux) at 32°C.

Results

Sporulation in liquid and solid media and the effect of temperature

Anabaena torulosa grew well in both Glade's and cyanophycean (figure 1) media. Sporulation occurred readily in Glade's liquid medium (figure 2c, 4c). Temperatures upto 40°C did not affect growth (figure 2a), but higher temperature (40°C) induced early sporulation (figure 2c), compared to relatively low temperatures (32°C, figure 2; 29°C, figure 4). Heterocyst frequency was also relatively higher at 40°C (figure 2b).

Although Glade's liquid medium was very effective in favouring sporulation (figure 2c; 4c) *A. torulosa* did not sporulate in liquid cyanophycean medium at
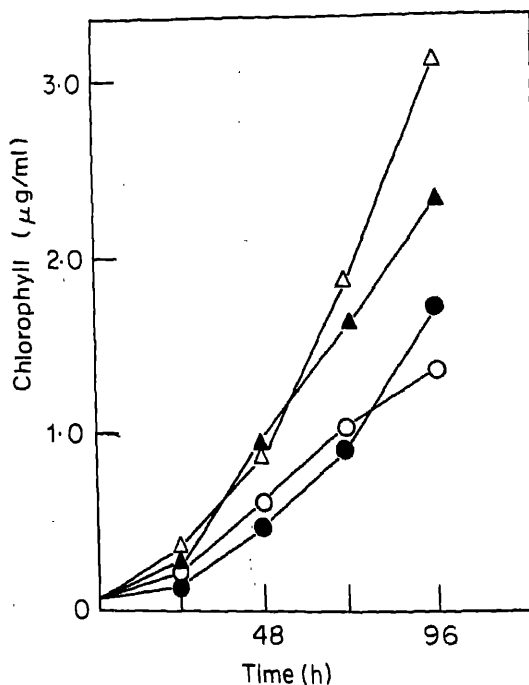
Figure 1. Growth and sporulation of *Anabaena torulosa* at 29°C in a minimal (C medium and a minerally enriched (cyanophycean) medium, in the presence of either inc cent (3000 lux) or fluorescent (3000 lux) light. Growth in Glade's meidum: incandesc and fluorescent (O) light. Growth in cyanophycean medium: incandescent (Δ fluorescent (●) light.
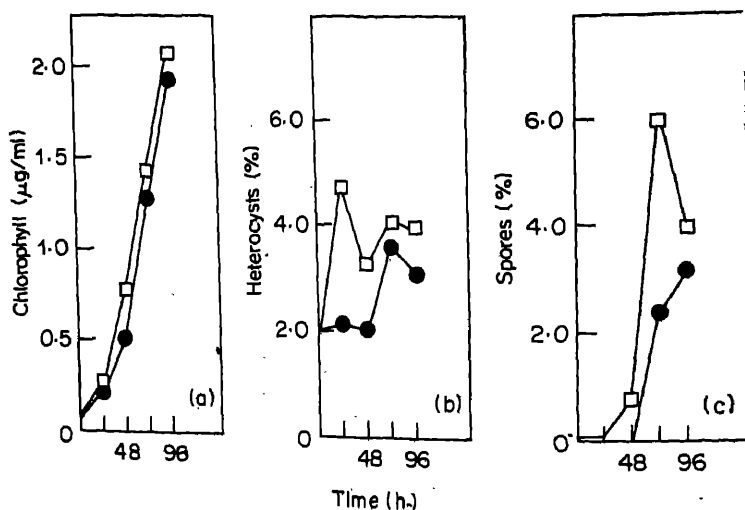
Figure 2. Effect of temperature on (a) growth, (b) heterocyst production and (c) spo tion of *Anabaena torulosa* in Glade's medium in the presence of incandescent light (3000 32°C (●); 40°C (□). The enhancement in sporulation at 40°C over that at 32°C statistically significant with L.S.D. values at p=0.05 level being 1.05, 1.35 and 0.78 for 48, 7. 96 h old cultures respectively.

lower (29 and 32°C) temperatures (figure 3b). Only at a higher temperature (37°C) there was indication of sporulation. However, when the alga was inoculated on solid (agar 1%, w/v) cyanophycean medium sporulation occurred readily (figure 3a) and was influenced greatly by temperature. On solid medium, sporulation at 37°C was several times that at 29°C and about two to three times as much as at 32°C.
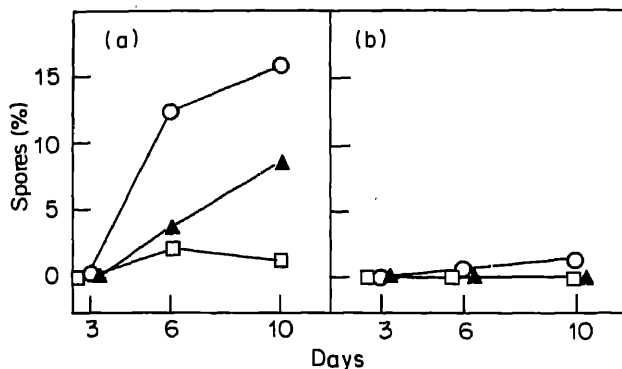
Figure 3. Sporulation of *Anabaena torulosa* in (a) solid (1% agar) and (b) in liquid cyanophycean medium at 29°C (□), 32°C (▲), and 3″ C′(O) in the presence of incandescent light (1000 lux).

Effect of light source and intensity on sporulation

Heterocyst frequency was not affected by the quality of light except in the late linear phase of growth, when more heterocysts occurred in incandescent light (figure 4b). The effect of light quality was most remarkable on the differentiation of spores. Whereas sporulation occurred readily after 3 days of growth at 29°C and reached nearly 7% on day 5 in incandescent light, there was no sporulation in fluorescent light (figure 4c).
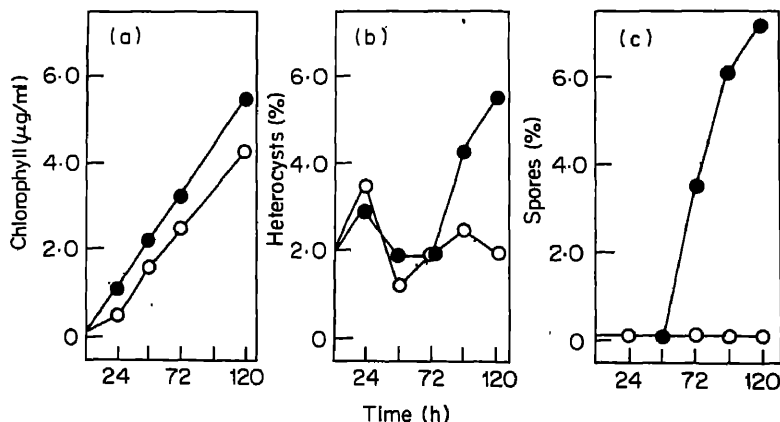
Figure 4. Effect of light source (3000 lux) on (a) growth, (b) heterocyst production and (c) sporulation of *Anabaena torulosa* in Glade's medium at 29°C. Incandescent light (●); fluorescent light (O).

Under incandescent light intensities of 1000, 3000 and 5000 lux heterocy$\cdot$ percentage was maintained near 4 during the growth period, but sporulation w$\jmath$ enhanced nearly in proportion to the light intensity used (table 1).

Table 1. Effect of light intensity on sporulation in *Anabaena torulosa*.

Light Intensity (lux)	Age of culture								
	48 h			72 h			96 h		
		LSD			LSD			LSD	
	spores %	p=0.05	p=0.01	spores %	p=0.05	p=0.01	spores %	p=0.05	p=0.09
1000	0.20			1.83			1.46		
3000	1.74	1.15	2.55	5.09	1.30	2.60	4.46	0.47	1.05
5000	4.49	0.47	1.10	6.00	0.21	0.65	7.40	0.70	1.45

Effect of phosphate on sporulation

Low phosphate concentration markedly enhanced sporulation (figure 5), thι incidence of sporulation being inversely related to phosphate concentration. Even in the minerally enriched cyanophycean medium, which did not generall$\mathfrak{s}$ favour sporulation, lack of phosphate induced spore formation. Low phosphatϵ concentration had no apparent effect on growth and heterocyst differentiati$\mathfrak{w}$r (figure 5).
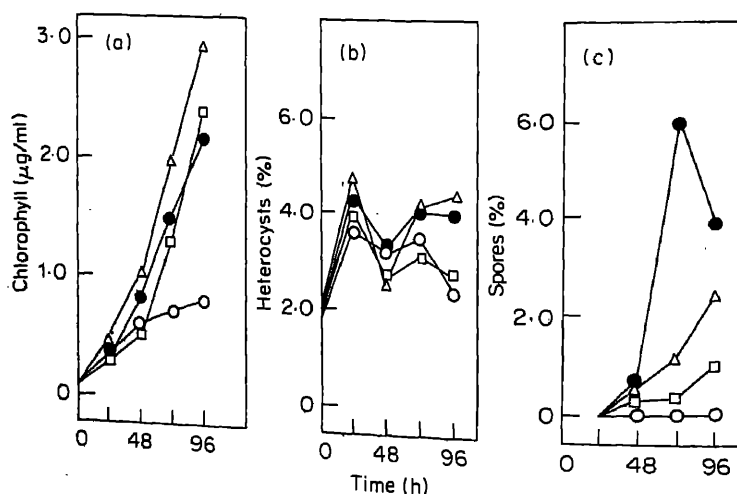
Figure 5. Effect of phosphate on (a) growth, (b) heterocyst production and (c) sporulation of *Anabaena torulosa* at 3000 lux incandescent light and at 29°C in Glade's medium containing 0.3 (●), 0.7 (△), 1.0 (□) and 1.4 (O) mM K_2HPO_4.

Extracellular spore-stimulating substance

When *Anabaena torulosa* was grown in the filtrate obtained from 8 day old cultures (see Methods), sporulation was greatly enhanced (table 2). Culture filtrate and fresh Glade's medium in the ratios 1:1, 1:3 and 1:5 also stimulated sporulation when

Table 2. Effect of culture filtrate on sporulation in *Anabaena torulosa.*

Ratio of culture filtrate to fresh medium[a] (v/v)	Age of culture (h)								
	48 h			72 h			96 h		
	spores %	LSD p=0.05	p=0.01	spores %	LSD p=0.05	p=0.01	spores %	LSD p=0.05	p=0.01
0 : 1	0.00			4.70			6.96		
1 : 0	5.80	0.33	0.57	12.19	0.74	1.30	13.21	1.15	2.00
1 : 1	3.30	0.32	0.56	9.42	0.65	1.15	10.80	0.98	1.75
1 : 3	0.99	0.12	0.21	6.60	0.72	1.25	9.06	1.20	2.25
1 : 5	0.50	0.05	0.08	5.80	0.98	1.70	7.20	1.15	2.05

[a] Filtrate of 8 day old cultures were diluted with fresh Glade's medium (see Methods).

compared to the efficiency in Glade's medium alone. Heterocyst frequency was not distinctly affected by the presence of culture filtrate. Although growth was clearly inhibited in the experiments with culture filtrate alone, dilution with fresh medium prevented the inhibition without significantly decreasing the stimulatory effect on sporulation.

Sequence of sporulation

When 6-day old *A. torulosa* grown in cyanophycean medium (non-sporulating) was inoculated into Glade's medium and grown at 32°C, spores differentiated normally within 48 h (table 3). In a typical experiment, after 41 h of growth 65% of the

Table 3. Sequence and pattern of sporulation in *Anabaena torulosa.*

Hours	Number of spore strings				Total cell number	
	S-H[a]	S-H-S[a]	S-S-H-S[a]	S-S-H-S-S[a]		
24	0	0	0	0	4948	(111)[b]
31	0	0	0	0	3576	(102)
41	104 (65.0)[c]	32 (20.0)[c]	0	0	18,042	(233)
48	125 (68.6)[c]	54 (29.6)[c]	0	0	16,310	(264)
72	155 (34.6)[c]	267 (59.6)[c]	26 (5.8)[c]	0	19,090	(268)
96	79 (16.0)[c]	332 (69.7)[c]	58 (12.2)[c]	7 (1.4)[c]	11,621	(148)

S-H, S-H-S etc. denote the pattern of spore string formation contiguous to heterocysts. S and H stand for spore and heterocyst respectively. Spores away from heterocysts were not found in the filaments examined in this experiment.

[b] Values in parenthesis are number of filaments examined.

[c] Values in parenthesis indicate per cent heterocysts having the specific type of spore string.

heterocysts had only one adjacent spore (figure 6a; spore—heterocysts—vege...
tive cell or S-H type, table 3) and 20% had a spore on either side of the heteros...
(figure 6b S-H-S type). The remaining heterocysts had no attached spore. I...
h, almost all heterocysts had attached spores and the percentages of S-H and S-H...
types were 69 and 30, respectively. At 72 h, the respective % values chang...
drastically to about 35 and 60 (table 3). In addition, nearly six % of the heteros...
had two spores on one side and a single spore on the other adjacent side (S-S-H...
type; figure 6c). By the end of the fourth day (96 h), the % of S-H type decrea...
further with concomitant increase in S-H-S and S-S-H-S types. Moreover, s...
heterocysts had also two spores on either side (S-S-H-S-S; figure 6d). These...
indicate that the sequence of sporulation pattern development was: S-H→S-H→
S-S-H-S→S-S-H-S-S.
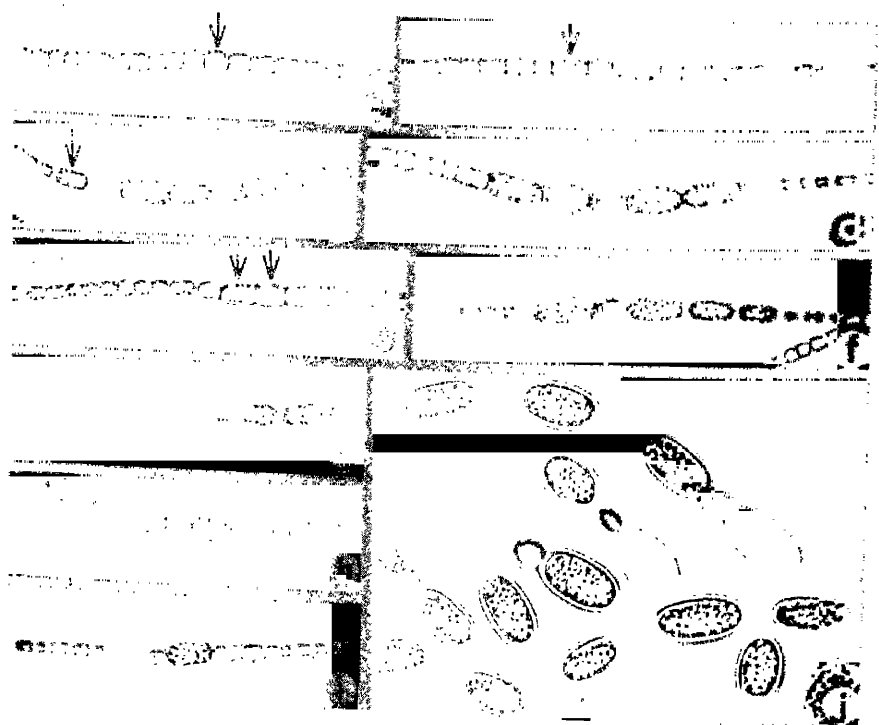
Figure 6. Phase contrast photomicrographs of *Anabaena torulosa* showing the sequence of formation of spore chains. Magnification: X-830. (a) Initiation of a spore adjacent to one side of a heterocyst (S-H type pattern) is followed by (b) spore formation on the other side (S-H-S type). Subsequently spore strings form in (c) S-S-H-S and (d) S-S-H-S-S pattern. Occasion- ally the pattern is more unilateral resulting in the formation of (e) S-S-H, (f) S-S-S-H-S, or (g) S-S-S-S-H-S spore strings. Rarely spores appear (h, i) several cells away from heterocysts. Filaments of old cultures lyse leaving behind large mature spores (j) often with their attachments to heterocysts intact. Arrows indicate spores at various stages of differentiation.

Rarely, the sequence occurred more unilaterally with spore strings forming only on one side of the heterocyst (S-S-H; figure 6e) resulting later in S-S-S-H-S (figure 6f) or S-S-S-S-H-S (figure 6g) patterns. Very rarely, spores were also initiated few or many cells away from heterocysts (figure 6h, i). After about five days' growth in Glade's medium, the cyanobacterial filaments broke up, releasing spores (figure 6j) often along with the heterocysts to which they were attached.

Discussion

In *A. cylindrica*, sporulation has been shown to occur mainly during the late logarithmic and stationary phases of growth (Simon, 1977). In *A. torulosa* under conditions favourable for sporulation, such as growth in a minimal (Glade's) medium and provision of incandescent light, spores are initiated even during the exponential phase. During this period, growth is not diminished in Glade's medium and is comparable to that in the minerally enriched cyanophycean medium. This indicates that the occurrence of sporulation is not an effect resulting from decreased growth rate.

Relatively high temperature is known (Wolk, 1965) to facilitate sporulation in *A. cylindrica*, although temperature above 30°C was not considered suitable because of deleterious effects on growth. In *A. torulosa* higher temperatures are found to favour sporulation and even a temperature of 40°C does not hamper growth and enhances sporulation.

The present results with *A. torulosa* showing enhanced sporulation in high light intensity are in contrast to the results with *A. cylindrica* (Wolk, 1965) where low light intensity has been shown to favour sporulation. However, it appears that spectral quality of the light is more important in sporulation than its intensity. In *Nostoc spumigena* red and blue light have been reported to stimulate sporulation (Pandey and Talpasayi, 1980). Our finding that incandescent light stimulates sporultion whereas no sporulation occurs in fluorescent light, suggests the possible involvement of red light in the differentiation of spores. The involvement of light quality on spore differentiation needs to be critically evaluated by using monochromatic light.

The spore stimulating activity under low phosphate concentrations confirms similar conclusions made earlier (Glade, 1914; Fisher and Wolk, 1976). Even in the minerally enriched cyanophycean medium, which does not normally favour sporulation, lack of phosphate induces spore formation. However, the role of phosphate does not seem pivotal, because sporulation of *A. torulosa* occurs readily in solid (agar) cyanophycean medium containing phosphate. It may be that unlike the situation in liquid cultures, extracellular substances may not get diluted in solid medium, and would therefore be present near algal filaments in amounts sufficient to induce sporulation. The involvement of extracellular products in sporulation has been demonstrated in *C. licheniforme* (Fisher and Wolk, 1976; Hirosawa and Wolk, 1979b) and the enhancement in sporulation observed by us in *A. torulosa* on addition of culture filtrates indicates that this involvement may be of general occurrence in cyanobacteria.

It has been suggested (Wolk, 1965) that a gradient of spore maturation exists in *Anabaena cylindrica* and an assessment of the rate of spore formation in this alga (Simon, 1977) supports this notion. Our results on the course of sporulation in *A. torulosa* also agree with the above conclusion. However, the pattern of sporulation ·in *A. torulosa* not only suggests a bilateral sequence emanating from the heterocyst, but also indicates that the impulse for sporulation is unequally manifested in that, spores are mostly initiated on one side first and not on both sides simultaneously. This is evident from the time course of initiation of spores on either side of the heterocysts and also from the unequal size of the spores on either side immediately contiguous to heterocysts.

The spatial relationship between heterocysts and spores in *A. cylindrica* is broken in the presence of canavanine suggesting that the physiological state of the vegetative cell, and not dependence on heterocysts is the major determining factor in sporulation (Nichols *et al.,* 1980). This is also suggested by the occasional appearance of spores away from heterocysts in *A. torulosa*. However, under normal conditions heterocysts seems to have a role in regulating spore formation, possibly by the production of.a substance which abates the inhibition apparently resident in vegetative cells (Wolk, 1965; Nichols *et al.,* 1980). Although there is no clear evidence as yet to confirm this possibility, the present results and those reported earlier (Wolk, 1965; Tyagi, 1974; Simon, 1977) favour the concept that gradients of spore maturation exist in cyanobacteria.

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Metabolism of glycosaminoglycans in rats during methionine deficiency and administration of excess methionine

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Abstract. Methionine deficiency in rats caused significant decrease in the concentration of many sulphated glycosaminoglycans in the aorta and other tissues, while administration of excess methionine caused an increase in these constituents. The activity of some important biosynthetic enzymes decreased in methionine deficiency and increased on administration of excess methionine. No uniform pattern was observed in the changes in the activity of enzymes concerned with degradation of glycosaminoglycans. The concentration of 3'-phosphoadenosine 5'-phosphosulphate and the activities of the sulphate activating system and sulpho-transferase were decreased in methionine deficiency, while feeding excess methionine did not affect these parameters as compared to controls.

Keywords. Methionine deficiency; glycosaminoglycans; sulphate-activating enzymes; sulpho-transferases.

Introduction

The quality and quantity of protein in the diet have been reported to affect the metabolism of glycosaminoglycans (GAG) (Leelamma and Kurup, 1978; Menon and Kurup, 1975). This effect may in part be related to the amino acid composition of the protein. So far there have been very few studies on the relationship of methionine to the metabolism of glycosaminoglycans. Udapa *et al.* (1956) reported a decreased accumulation of mucopolysacharides during wound healing in rats on protein free diet and when supplemented with methionine, the accumulation and subsequent disappearance of mucoplysacharides were normal. Srinivasan *et al.* (1972) showed that methionine prevented the decrease in intestinal mucopolysccharides in rats caused by ethionine feeding.

Excessive amounts of sulphur containing aminoacids (methionine and cysteine) have been reported to cause accumulation of liver lipids (Aoyama *et al.*, 1969; Aoyama *et al.*, 1978). It is known that the lipid accumulation in the aorta is the result of interaction between serum lipoproteins (LDL and VLDL) and GAG in the aorta (Iverius, 1973) and depends on the concentration of aortic GAG as well as that of serum LDL plus VLDL. It was therefore considered necessary to study the

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Abbreviations used: GAG, glycosaminoglycans; VLDL or LDL, VDL, very low, or low or high density lipoproteins; PAP, 3'-phosphoadenosine-5' phosphosulphate.

metabolism of GAG in rats fed with methionine-deficient and methionine-excess diets. The concentration of different GAG fractions in the aorta and other tissues, and the activities of some key enzymes such as glucosamine phosphate isomerase UDPG-dehydrogenase, β-glucuronidase, β-N-acetyl glucosaminidase; hyaluronoglucosidase, as well as sulphation have been studied. The concentration of cholesterol in serum HDL and LDL plus VLDL and that of cholesterol and triglycerides in the aorta have also been studied in rats fed on excess methionine containing diet. The results are reported in this paper.

Materials and methods

Male albino rats (Sprague-Dawley strain, body weight 80-100 g) were divided into 4 groups as in table 1, with 15 rats in each group.

Table 1. Diet formulation (in g)

Group	Dextrin	Amino acid mixture (without methionine)	Casein (fat free)	Groundnut oil	Salt mixture*	Vitamin* mixture	Methionine
Methionine deficient	69.7	17.3	—	8.0	4.0	1.0	0.027
Control, pair-fed to Group 1	69.2	17.3	—	8.0	4.0	1.0	0.54
Methionine-adequate	71.0	—	16.0	8.0	4.0	1.0	—
Methionine-excess	70.5	—	16.0	8.0	4.0	1.0	0.54

Composition of the amino acid mixture (g) (Correspond to that present in 16% casein Hawk's physiological chemistry, ed. Oser (1965):

L-Histidine—0.5, L-valine—1.15, L-glutamic acid—3.58, L-Leucine—1.47, L-tryptophan—0.14, L-lysine—1.3, L-threonine—0.78, L-aspartic acid—1.14, L-tryosine—1.10, L-serine—1.0, L-glycine—0.43, L-phenylalanine—0.8, L-proline—1.81, L-alanine—0.48, L-arginine—0.65, L-cystine—0.048 and L-isoleucine—0.98.

* Menon and Kurup (1976)

The methionine-deficient group thus received only 1/20th of the amount of methionine given to the control group. Since dietary consumption in the methionine-deficient group was lower, the control group (group 2) was fed the same amount of methionine-adequate diet so as to maintain a comparable caloric intake to facilitate comparison between these two groups.

The methionine-excess group received 100% more methionine than the adequate diet group. The dietary consumption was more or less the same among the rats of groups 3 and 4.

The rats in both experiments were maintained on their respective diets for a period of 14 days.

At the end of this period, the rats in each group were deprived of food overnight, stunned and killed by decapitation. The tissues were quickly removed to ice cold containers for various estimations.

Estimation of lipids

Extraction of the tissues for lipds and estimation of cholesterol and triglycerides were carried out as described before (Sandhyavathy Bai and Kurup, 1976). Serum HDL, LDL and VLDL were separated by the heparin-manganese precipitation method described by Warnick and Albers (1972).

Estimation of glycosaminoglycans

GAG were estimated in the tissues as described before (Sudhakaran and Kurup, 1974). Papain digest of the defatted tissue was passed through a column of cellulose and the different fractions-hyaluronic acid, heparin sulphate, chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate and heparin were eluted according to the procedure of Svejcar and Robertson (1967). The individual fractions were quantitated by the estimation of uronic acid by the method of Bitter and Muir (1962). The identity of the GAG fractions in each case was confirmed by comparison with standard GAG preparations. (Made available by the courtesy of Prof. M. B. Mathews, University of Chicago, Chicago, Illinois, USA).

Estimation of enzyme activities

The activity of glucosamine phosphate isomerase (glutamine forming) (EC 5.3.1.19) was estimated by the method of Pogell and Gryder (1957) and that of UDPG dehydrogenase (EC 1.1.1.22) by the method of Strominger *et al.* (1957). β-Glucuronidase (EC 3.2.1.31), β-N-acetyl glucosaminidase (EC 3.2.1.30) and hyaluronoglucosidase (EC 3.2.1.35) were estimated according to the procedure of Kawai and Anno (1971) and Cathepsin-D (EC 3.4.23.5) by using 4% haemoglobin in 0.1 M acetate buffer PH 4.5 as the substrate and determining the amount of tyrosine liberated by the method of Folin and Ciocalteu (1927).

Sulphate metabolsim

The estimation of the concentration of 3'-phosphoadenosine-5'-phosphosulphate (PAPS) and the activity of sulphate activating system [which includes sulphate adenyl transferase (EC 2.7.7.4) and adenyl sulphate kinase (EC 2.7.1.25)] and aryl sulphotransferase (EC 2.8.2.1) of the liver were carried out as described before (Sudhakaran and Kurup; 1974) according to the procedure of Vankempen and Jansen (1972, 1973) using methyl umbelliferone.

Protein was estimated in the enzyme extract after trichloroacetic acid precipitation by the method of Lowry *et al.* (1951).

Statistical analysis

The data given in the tables are the mean for the number of animals used in each case ±SEM. Statistical significance was calculated using Students' t-test (Bennet and Franklin, 1967).

Results and discussion

Methionine-deficient rats showed lower gain in body wt. (8.2 ±1.9 g) as comp
to the corresponding pair-fed control (21.6 ±2.4 g). The gain in body wt. ii
methionine-excess group (16.8 ±2.9 g) was also lower than that in the correspon
control group (23.6 ±2.2 g).

Concentration of cholesterol and triglycerides in the serum and aorta in rats
with excess methionine

There was significant increase in the concentration of cholesterol and triglyceri
in rats fed with excess methionine diet as compared to those fed with just
adequate dose. HDL cholesterol showed significant decrease in these rats wh
LDL plus VLDL cholesterol increased (table 2).

Table 2. Concentration of cholesterol and triglycerides in serum and aorta in rats fed with
excess methionine diet.

Groups	Cholesterol				Triglycerides (as glycerol)	
	Serum	HDL	LDL+VLDL	Aorta	Serum	Aorta
3	73.1 ±2.92	50.16 ±2.0	22.94±0.99	187.08 ±8.2	6.35 ±0.23	614.5 ±27.5
4	125.4^a±5.02	29.6^a ±1.33	95.4 ±4.29	273.57^a±11.01	8.2^b ±0.4	1144.5^a±51.5

Average of the values from 6 rats in each group ±SEM, values expressed as mg/100 g wet tissue
except for serum where it is mg/100 ml ±SEM

^{a}p less than 0.01
^{b}p between 0.01 and 0.05
HDL—high density lipoprotein
LDL—low density lipoprotein
VLDL—very low density lipoprotein.

Concentration of GAG and activity of some enzymes of GAG metabolism in the
aorta

In the methionine deficient group, there was a significant decrease in chondroitin-4
and -6 sulphate in the aorta as compared to the pair-fed control group, while there
was no significant alteration in heparin, heparin sulphate and dermatan sulphate.

However, an increase in hyalauronic acid content was observed. On the other
hand, rats fed with excess methionine diet showed significant increase in all the
GAG fractions in the aorta except heparin, as compared to the corresponding
control group. Heparin was not significantly altered (table 3).

Glucosamine-6-phosphate isomerase activity decreased in methionine-deficient
rats as compared to the pair-fed control but in rats fed with excess methionine diet,
the enzyme activity increased significantly.

Table 3. Metabolism of glycosaminoglycans in the aorta.

Groups	Concentration of GAG*						Activity of enzymes				
	HA	HS	Ch-4S	Ch-6S	DS	H	Glucosamine phosphate[c] (isomerase)	Hyaluronidase[d]	Glucuronidase	Hexosaminidase	Cathepsin D[e]
1.	$1090.6^a \pm$ 32.7	$1504.8 \pm$ 45.12	$706.4^a \pm$ 21.18	$1029.6^a \pm$ 30.6	$914.8 \pm$ 27.4	$896.8 \pm$ 26.9	$19.42^a \pm$ 0.6	$113 \pm$ 3.4	$61.92 \pm$ 1.86	$72.46^a \pm$ 2.17	$113.7^a \pm$ 3.41
2.	$760.39 \pm$ 22.8	$1600.46 \pm$ 48	$908.92 \pm$ 27.24	$1260.39 \pm$ 37.8	$984.85 \pm$ 29.5	$908.69 \pm$ 27.2	$28.6 \pm$ 1.0	$117 \pm$ 3.5	$65.38 \pm$ 1.96	$86.27 \pm$ 2.59	$137.9 \pm$ 4.14
3.	$776.5 \pm$ 31.04	$1609.3 \pm$ 64.4	$912.92 \pm$ 36.52	$1314.32 \pm$ 56.3	$963.4 \pm$ 38.5	$915.17 \pm$ 36.6	$27.3 \pm$ 0.96	$121.2 \pm$ 3.64	$63.23 \pm$ 1.89	$87.1 \pm$ 2.61	$139 \pm$ 4.17
4.	$900.67^b \pm$ 36.0	$2034.9^a \pm$ 81.4	$1445.34^a \pm$ 57.8	$2525.58^a \pm$ 101	$1209.8^a \pm$ 48.4	$891.7 \pm$ 15.6	$38.14^a \pm$ 1.33	$128.52 \pm$ 3.86	$85.21^a \pm$ 2.56	$93.83 \pm$ 2.81	$158.23^b \pm$ 4.75

Average of the values from 6 rats in each group ± SEM.
Group 1 has been compared with group 2.
Group 4 has been compared with group 3.
HA—hyaluronic acid; HS—heparin sulphate; CH4S—chondroitin-4 sulphate; Ch-6S—chondroitin-6 sulphate; D—dermatan sulphate;
H—heaparin.
[a] p less than 0.01
[b] p between 0.01 and 0.05
[c] μmol of 3 hexosamine/h/g protein ±SEM
[d] mg of N-acetyl hexosamine/h/g protein ±SEM
[e] mg of p-nitrophenol/h/g protein ±SEM
[f] mg of tyrosine liberated/h/g protein ±SEM

* μg uronic acid/g dry defatted tissue.

Table.4. Metabolism of Glycosaminoglycans in the liver.

a) Concentration of GAG fractions*						b) Activity of Enzymes					
Groups	HA	HS	Ch-4S	Ch-6S	DS	H	Isomerase	UDPG dehydrogenase	Hyaluronidase	Glucuronidase	Hexosaminidase	Cathepsin D
1.	$114.84^a \pm$ 3.4	$208.7^a \pm$ 6.2	$133.2 \pm$ 3.42	$114.8^a \pm$ 3.42	$139.62 \pm$ 4.17	$110.8^a \pm$ 3.3	$34.49^a \pm$ 1.2	$2260.54 \pm$ 79	$70.2 \pm$ 2.1	$65.14 \pm$ 1.95	$58.61^b \pm$ 1.76	$147.96 \pm$ 4.43
2.	$140.7 \pm$ 4.2	$269.32 \pm$ 8.1	$147.8 \pm$ 4.4	$171.08 \pm$ 5.13	$147.57 \pm$ 4.41	$191.3 \pm$ 5.73	$43.58 \pm$ 1.53	$2369.8 \pm$ 82.92	$67.9 \pm$ 2.03	$72.6 \pm$ 2.18	$66.16 \pm$ 1.98	$161.8 \pm$ 4.85
3.	$149.0 \pm$ 4.47	$273.2 \pm$ 10	$154.3 \pm$ 6.2	$178.6 \pm$ 6.23	$153.1 \pm$ 6.2	$200.09 \pm$ 7.0	$36.5 \pm$ 1.3	$2338.56 \pm$ 81.86	$66.85 \pm$ 2.0	$73.12 \pm$ 2.19	$65.43 \pm$ 1.98	$162.09 \pm$ 4.86
4.	$160.8^b \pm$ 4.82	$316.8^b \pm$ 9.5	$231.4^a \pm$ 9.2	$252.17^a \pm$ 8.8	$176.8 \pm$ 6.96	$176.5 \pm$ 6.2	$51.7^a \pm$ 1.8	$3584.1^a \pm$ 125.46	$98.32^a \pm$ 2.9	$79.8 \pm$ 2.39	$81.86^a \pm$ 2.46	$188.96 \pm$ 5.6

* 1 μg uronic acid/g dry defatted tissue ±SEM;

Foot note same as for table 3.

Hyaluronoglucosidase and β-glucuronidase activities were not significantly altered in the methionine-deficient rats while β-N acetyl glucosaminidase and Cathepsin D activity decreased. In rats fed with excess methionine diet the activities of Cathepsin-D and β-glucuronidase increased in the aorta while those of β-N-acetyl glucosaminidase and hyaluronoglucosidase were not significantly altered.

The aorta is particularly important both from the point of view of lipid accumulation in atherosclerosis and the high concentration of GAG present. Excess methionine caused increase in LDL plus VLDL cholesterol in the serum and in aortic cholesterol and triglycerides. It is known that serum lipoproteins (LDL plus VLDL) interact with aortic GAG forming insoluble complexes and this interaction has been considered to be responsible for lipid accumulation in the arterial wall (Iverius 1973). This interaction involves molecular seiving effect (Laurent *et al.,* 1963; Iverius, 1970), stearic exclusion effect (Laurent, 1968; Iverius, 1968) and ionic interaction (Srinivasan *et al.,* 1972) and has been well studied. The decreased concentration of many GAG fractions in the aorta in methionine deficiency may result in decreased interaction with the serum lipoproteins with consequent decrease in lipid accumulation in the aorta. Decreased lipid accumulation has been reported in tissues in methionine deficiency (Aoyama and Ashida, 1972; Aoyama *et al.,* 1969). In rats fed with excess methionine diet, the increased concentration of GAG in the aorta, along with the increase in serum LDL plus VLDL may result in their increased interaction resulting in increased accumulation of lipids in this tissue. The increased concentration of cholesterol and triglycerides in the aorta obtained in rats fed with excess methionine is in support of this view.

The synthesis of GAG is regulated by glucosamine phosphate isomerase as well as UDPG dehydrogenase. The isomerase provides glucosamine-6-phosphate which is the precursor of hexosamines in GAG synthesis, while UDPG dehydrogenase makes available UDP glucuronic acid, the precursor of uronic acid moieties. The activity of the former enzyme is regulated by feed back inhibition by UDP-N-acetylglucosamine and that of the latter by UDP-xylose. UDPG dehydrogenase activity could not be studied in the aorta but the decreased activity of the isomerase in methionine deficient rats would result in decreased availability of hexosamine precursors for GAG synthesis. The decreased concentration of GAG in the aorta in methionine deficiency may therefore be due to their decreased synthesis. In rats fed with excess methionine, the isomerase activity is increased making more glucosamine-6-phosphate available for GAG synthesis but in the absence of information on the activity of UDPG dehydrogenase, it is not possible to speculate whether the synthesis of GAG is increased in the aorta in this case.

The pattern of change in the GAG degrading enzymes is not uniform in the aorta. In methionine deficiency, the activities of some enzymes decreased while those of others were not significantly altered. In methionine-excess group, some enzyme activities increased while the activities of others were not significantly altered. Since the concerted action of all these enzymes would be required for the degradation of GAG, it is not possible to say how the overall degradation of GAG is affected by methionine deficiency or administration of excess methionine.

Concentration of GAG and activity of some enzymes of GAG metabolism liver

The concentration of hyaluronic acid, heparin sulphate, chondroitin-6 and heparin was decreased in the liver in methionine deficiency while chon 4-sulphate and dermatan sulphate were not altered. In rats fed excess meth heparin sulphate, chondroitin-4 and -6 sulphate and dermatan sulphate in while heparin and hyaluronic acid were not significantly altered. (Table 4

The activity of glucosamine phosphate isomerase decreased in the liver in r nine deficiency while UDPG dehydrogenase was not affected. Both these e activities increased in the liver in rats fed excess methionine. The dec activity of isomerase in the liver in methionine deficiency even though dehydrogenase was not affected, may result in decreased synthesis of GAG in tissues and the decreased concentration of many GAG observed in meth deficiency may be due to this. The increased activity of both these enzymes liver in rats fed excess methionine may result in increased GAG synth agreement with the increased concentration of GAG observed.

Activity of hyaluronoglucosidase, cathepsin D and β-glucuronidase w affected in methionine deficiency while that of β-N-acetyl glucosamin decreased. But in rats fed excess methionine the activities of cathepsin D acetyl glucosaminidase and hyaluronoglucosidase increased while that glucuronidase was not affected. Thus, the pattern of change in these en activities in the liver, as in the case of aorta, is not uniform and therefore it possible to say how the degradation of GAG is affected in the liver by methion

Sulphate metabolism

Methionine deficiency resulted in significant decrease in the concentratio PAPS in the liver as also in the activity of sulphate activating system and su transferase. But feeding excess methionine did not alter these parameters their normal values. The decrease in the concentration of PAPS in methio deficiency is due to the decrease in the activity of sulphate activating sy (including sulphate adenyl transferase and adenyl sulphate kinase) which gene PAPS. Decreased sulphate metabolism can result in decreased sulphatio glycosaminoglycans. The decrease in some of the sulphated GAG observe methionine deficiency in the liver may also be due to the decreased sulp metabolism. Even though the sulphotransferase now studied is aryl-sulphotr ferase, PAPS is also the sulphate donor for sulphation of GAG. Thus decreased concentration of PAPS would affect sulphation of GAG also. possible that methionine also provides sulphate for the formation of PAPS an its deficiency, sulphate metabolism is decreased (table 5).

Changes in the concentration of GAG in the heart and kidney

In methionine deficient rats, hyaluronic acid, heparin sulphate and chondroit sulphate decreased in the heart while chondroitin-4 sulphate, dermatan sulph and heparin were not significantly altered. In the kidney, only hyaluronic a showed decrease while other GAG fractions were not affected.

Table 5. Concentration of PAPS, activity of sulphate activating system and Sulphotransfer-ase in the liver of rats fed with methionine deficient and methionine excess diet.

	PAPS*	Sulphotransferase*	Sulphate activating system*
1)	145.3[a] ±4.4	16.50[a]±0.5	15.83[a]±0.48
2)	173.67 ±5.21	22.8 ±0.68	27.23 ±0.82
3)	167.8 ±5.03	21.5 ±0.65	26.5 ±0.8
4)	159.3 ±4.78	20.76 ±0.62	24.65 ±0.74

* µmol of methylumbellifuone sulphate formed/h/g protein.

Foot note same as for table 3.

On the other hand, rats fed excess methionine showed increase in chondroitin-4 sulphate, chondroitin-6 sulphate and dermatan sulphate in the heart and kidney, Heparin sulphate increased in the heart but was not affected in the kidney. Hyaluronic acid increased in the kidney but was not altered in the heart. Heparin decreased in the heart, but was not altered in the kidney.

Table 6. Concentration of glycosaminoglycans in the heart and kidney.

Groups	H.A.	H.S.	FRACTIONS Ch-4S	Ch-6S	DS	H
Heart						
1.	137.39[a]± 4.1	267.27[b]± 8.1	196.8 ± 5.9	171.15[a]± 5.13	132.06 ± 3.96	127.84 ± 3.8
2.	218.43 ± 6.5	312.31 ± 9.4	215.44 ± 6.5	260.2 ± 7.8	132.3 ± 3.97	125.6 ± 3.75
3.	222.3 ± 8.9	314.6 ± 12.6	224.2 ± 8.9	272.3 ± 10.9	141.7 ± 5.64	129.01 ± 5.2
4.	205.4 ± 8.2	464.86[a]± 18.6	350.2[a]± 14	393.35[a]± 15.7	174.5[b]± 6.96	109.8[b]± 4.4
Kidney						
1.	319.7[a]± 9.6	355.8 ± 10.6	187.5 ± 5.91	276.9 ± 8.28	220.7 ± 6.6	312.5 ± 9.4
2.	384.8 ± 10.4	369.3 ± 11.07	207.8 ± 6.2	284.2 ± 9.8	232.14 ± 9.7	298.8 ± 8.9
3.	390.6 ± 13.7	374.5 ± 14.98	214.3 ± 8.57	296.2 ± 11.8	276.8 ± 9.6	284.6 ± 11.4
4.	474.8[a]± 14.2	430.63 ± 17.2	326.5[a]± 13.04	391.2[a]± 15.6	379.65[a]± 15.2	276.8 ± 11.04

Average values from 6 rats in each group ±SEM. Comparison of groups same as in table 3.
[a] P<0.01 [b] P between 0.01 and 0.05.
Values expressed as mg of uronic acid/g dry defatted tissue ±SEM) Foot note same as for table 3.

Thus metabolism of GAG is significantly affected by the level of methionine in the diet. This effect is seen at all levels viz., biosynthesis of precursors. degradation of GAG and sulphation.

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erties and characteristics of an anti-human
ionic gonadotropin monoclonal antibody

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Abstract.   The product of a hybrid cell clone, P_3W_{80}, obtained as ascites fluid from mouse peritoneal cavity had high titres of anti-human chorionic gonadotropin antibodies e.g. 30 to 40% binding of ^{125}I-human chorionic gonadotropin at 10^7 dilution in a radioimmunoassay. The antiserum SB_6 (raised against β-human chorionic gonadotropin distributed by National Institutes of Health, USA gave similar binding at 5000 dilution in parallel runs. The monoclonal antibody recognized best human chorionic gonadotropin (0.3 mIU of hormone/tube with $B/B_0 < 75\%$), but also bound β and α subunits of human chorionic gonadotropin, 12 and 800 folds lower than human chorionic gonadotropin respectively. No binding was observed with carboxy terminal peptides of β-human chorionic gonadotropin ranging from 93 to 145 amino acid residues, indicating the lack of recognition of the C-terminal region. No cross-reaction with human leutinizing hormone was obtained at the physiological surge levels, a significant competition ($B/B_0 < 75\%$, obtainable only at 60 mIU of LER 960 human leutinizing hormone/ tube. The antibody had heavy chain of IgG_1 and light chain of kappa type. It neutralized the bio-activity of human chorionic gonadotropin both *in vitro* and *in vivo*.

Keywords.   Human chorionic gonadotropin; monoclonal anti-hCG antibody; radioimmuno-assay; biological neutralization.

iction

aitis *et al.* (1972) obtained an antiserum by immunization of rabbits with beta of human chorionic gonadotropin (β-hCG) which had a high specificity for chorionic gonadotropin (hCG) and permitted the estimation of this ie in the presence of levels of hLH encountered in the serum. This anti-SB_6 has served as a valuable reagent for the estimation of hormone levels research on the hormone function (Goldstein *et al.*, 1974; Braunstein *et al.*, Subsequently anti-sera were raised against synthetic carboxy-terminal s of β-hCG which were totally devoid of cross-reaction with human ing hormone (hLH) (Chen *et al.*, 1976; Ramakrishnan *et al.*, 1979). r, due to low association constant of the antibodies for hCG, the sensitivity

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of their assay was one order lower for radioimmunoassay of HCG than SB₆ (Che
al., 1980). The anti-carboxy terminal peptides of βhCG sera also failed to neu
lize the bio-activity of hCG *in vivo* (Louvet *et al.,* 1974; Matsuura *et al.,* 1976)

We describe here the properties of a monoclonal antibody derived by hybrid
tion of mouse myeloma cells with splenocytes obtained from mice immunized
highly purified hCG. This antibody resembles SB₆ in several of its characteris
and is obtainable in very high titres and unlimited amount. In contrast to
antibodies raised against carboxy terminal peptides of βhCG, it neutralizes the
activity of hCG *in vivo*. This antibody, though highly specific for hCG does
bind with carboxy terminal peptides of βhCG, which is suggestive of
presence within the core of hCG of epitopes unique to the hormone in immu
reactivity.

Materials and methods

Hormones and peptides

Human chorionic gonadotropin (hCG; 10,000 IU/mg) was made available by
Tsong of the Population Council, New York, USA. Human luteinizing horm
(hLH; LER 960) was a generous gift from National Institutes of Health, Bethe.
Maryland, USA. Human prolactin (hPRL) was supplied by WHO under Qu₂
Control Programme. β-subunit of hCG (CR-119) and α-subunit (CR-119) v
made available by Drs S. Birken and R. E. Canfield of Columbia University, 1
York, USA. Carboxy-terminal peptides of β-hCG; 93-145; 111-145 and 115
conforming to the sequence proposed by Morgan *et al.* (1975), were preparec
Dr. Karl Folkers of Texas University and made available through the Internatic
Committee for Contraception Research (ICCR) of the Population Council, 1
York, USA. Alkaline phosphatase-tagged goat anti-mouse μ, G_1, G_{2a}, G_{2b} G
and λ chain-specific antibodies were obtained from Dr John Kearney, Universit
Alabama in Birmingham, Birmingham, Alabama, USA.

Borate buffered saline This was prepared by dissolving 6.185 g of boric acid,
g of sodium borate and 4.385 g of sodium chloride in 1 litre of double disti
water.

Alkaline phosphatase substrate buffer It was prepared by dissolving 2.45
$MgCl_2$ in 40 ml of distilled water containing 4.8 ml of diethanolamine (Sig
Chemicals, St. Louis, Missouri, USA). The pH of the solution was adjusted to
with 5N HCl and the volume made up to 50 ml with distilled water. *p*Aminophe:
phosphate (Biochemical Unit, V. P. Chest Institute, Delhi, India) was dissolve
the ethanolamine buffer (1 mg/ml) fresh before use.

Monoclonal antibodies

Hybridomas were prepared by fusion of spleen cells obtained from h
immunized mice with P3-NSI/1-Ag4-1 (NSI) a non-secreting variant of P3-X63-₂
(cell line of Balb/c origin derived from MOPC-21) as described elsewhere (Gu
and Talwar, 1980). Hybrids positive for anti-hCG antibodies were cloned
limiting dilution technique. Out of 110 clones thus developed, 10 were studied
their binding to iodinated gonadotropins as described elsewhere (Gupta :

Talwar, 1980). One of the positive clones, namely, P_3W_{80} was grown in the intraperitoneal cavity of the Pristane (Aldrich Chemical Co., Milwaukee, Wisconsin, USP) primed Balb/c mouse as ascites (Gupta and Talwar, 1980). Ascites fluid taped from intraperitoneal cavity was made cell-free by centrifugation at 800g for 15 min at 4°C. Subsequently, it was heat-inactivated at 56°C for 30 min, centrifuged at 15,000 g to remove debris, diluted with an equal volume of 10 mM phosphate buffer, pH 7.4 and lyophilized in aliquots of 0.5 ml. It was reconstituted in distilled water and subsequent dilutions were made in 10 mM phosphate buffer pH 7.4 containing 0.1% sodium azide and 0.1% bovine serum albumin for radioimmunoassay. For biological neutralization studies, the dilutions were made in isotonic saline containing 0.1% bovine serum albumin.

Specificity Studies: The reactivity of the ascites fluid obtained from P_3W_{80} clone of hybrid cells with various hormones was determined by competitive immunoassay. Iodination of hCG with carrier-free $Na^{125}I$ (Radio chemical Centre, Amersham, UK) was carried out by the method of Greenwood *et al.* (1963) as adopted by Vaitukaitis *et al.* (1972). The assay system contained 20 µl normal horse serum, 50 µl ^{125}I-hCG (100 to 150 pg), 50 µl solution of different hormones at varying concentrations and 50 µl of SB_6 or P_3W_{80} ascites fluid at the appropriate dilution to give 30 to 40% binding in absence of competition. Incubation of the assay mixture was carried out directly at 4°C for 18 to 20 h. The bound and free labelled hormone was separated by the addition of 1.0 ml of ammonium acetate-alcohol mixture as described by Salahuddin *et al.,* 1976.

Neutralization of biological activity of hCG

The mouse Lyedig cell bioassay system was used to investigate the ability of ascites fluid to neutralize the biological activity of hCG *in vitro*. To precipitate immunoglobulins, 0.2 ml of 30% polyethylene glycol solution was added to an equal volume of ascites fluid, vortexed and incubated overnight at 4°C. It was centrifuged at 1500 g for 15 min, supernatant discarded and the pellet was dissolved in 0.2 ml of phosphate buffer (pH 7.4). hCG (384 µIU) in 0.1 ml of phosphate buffer (pH 7.4) was preincubated with increasing dilutions of the precipitated immunoglobulins for 2 h at 37°C and subsequently at 4°C for 18 h. Leydig cell suspension was added to the preincubated hormone and the inhibition in the production of testosterone in presence of antibodies was estimated (Das *et al.,* 1978). The monoclonal antibodies were also tested for neutralization of the bioactivity of hCG *in vivo* by the mouse uterine weight gain assay. Prepubertal female mice of Balb/c strain, 20-21 days were given subcutaneously a total dose of 0.5 IU of hCG dissolved in 0.3 ml of isotonic saline with 0.1% bovine serum albumin in three equally divided daily doses. Ascites fluid (0.1 ml) at the indicated dilutions was also given subcutaneously every day in test mice at a site different from hCG.

Characterization of heavy and light chains of antibody secreted by P_3W_{80} clone

Alkaline phosphatase tagged goat anti-mouse µ, G_1, G_{2a}, G_{2b}, G_3, k and λ chain-specific antibodies were used in solid phase immunoassay to detect the chain specificity of the monoclonal antibodies. Essentially, the methodology described by Kerney *et al.* (1979) was followed except that polyvinyl microelisa plate (96 wells)

were coated with 100 µl of hCG solution (100 µg/ml in distilled water). At the end of the assay the reaction product of the wells were diluted with 0.3 ml of borate buffered saline and absorption of the reaction product was measured at 440 nm using a Pye-Unicam Spectrophotometer SP8-100.

Results

Reactivity of monoclonal antibodies with hCG and its comparision with SB.

The mouse ascites fluid containing the product of P_3W_{80} clone had a binding capacity of 30 to 40% of ^{125}I-hCG at a dilution of 10^7 in RIA. Using the same assay system, SB_6 gave 30-40% binding at 5000 dilution. hCG competed well with labelled hCG for binding to monoclonal antibody tested at 10^7 dilution (figure 1).
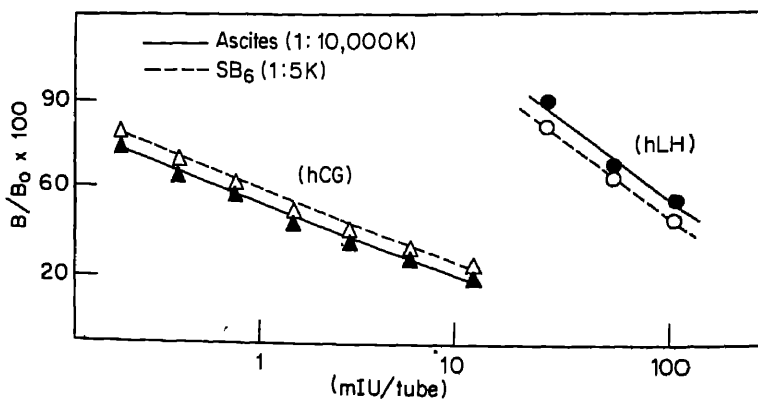
Figure 1. Comparative competitive inhibition profile of hCG and hLH.

The assay was done in a radioimmunoassay system using ^{125}I-hCG as tracer with monoclonal anti-hCG antibodies (—; P_3W_{80}) or SB_6 serum (— — — —). SB_6 was used at 5×10^3 dilution in the assay. hCG (P_3W_{80}), (▲); hCG (SB_6), (△); hLH (P_3W_{80}), (●); hLH (SB_6), (○). B=radioactivity bound to the antibody in presence of labelled hCG and unlabelled hormone. B_0=radioactivity bound with labelled hCG alone.

As low as 0.3 mIU of hCG per tube gave a significant inhibition of B/B_0 (i.e. <75%). With SB_6 0.45 mIU of hCG produced a similar inhibition. The slopes of SB_6 and monoclonal were by and large parallel. This monoclonal antibody was able to recognise hCG standards prepared either in male undiluted urine or serum (diluted 1:10 in 0.05 M phosphate buffer, pH 7.4 containing 0.1% bovine serum albumin and 0.1% sodium azide). The slopes and the amount of hCG required for ED_{50} (i.e. B/B_0=0.5; B=radioactivity bound to the antibody in presence of labelled hCG and unlabelled hormone; B_0=radioactivity bound with labelled hCG alone.) for this antibody as compared to the standard NIH antiserum SB_6 are given in table 1. The amount of hCG required for ED_{50} in urine (1.97 mIU/tube) was higher as compared to buffer (0.78 mIU/tube) and serum (0.93 mIU/tube). In all the three systems e.g. phosphate buffer, urine and serum, monoclonals were more efficient in assay of hCG as compared to SB_6. On a weight basis, the recognition capability of monoclonals was less (12 fold lower) for β-hCG as compared to the intact hCG and was still lower for α-hCG (800 fold) as compared to hCG (figure 2). SB_6

Table 1. Comparison of radioimmunoassay characteristics of SB_6 and the product of the clone P_3W_{80}

Antiserum	RIA characteristics					
	Buffer		Urine		Serum	
	Slope (±S.D.)	ED$_{50}^*$	Slope (±S.D.)	ED$_{50}^*$	Slope (±S.D.)	ED$_{50}^*$
SB_6	−2.42±0.13	1.18	−2.65±0.11	2.09	−2.34±0.12	1.57
P_3W_{80}	−2.60±0.19	0.78	−3.05±0.10	1.97	−2.40±0.20	0.93

Competitive binding displacement by hCG of ^{125}I-hCG binding to SB_6 and P_3W_{80} ascites fluid was studied. Slope was calculated by the Linear Regression formula $Y = mx + C$ where $Y = Log\ B/B_0$ (B:cpm bound in the presence of ^{125}I-hCG and unlabelled hCG; B_0: cpm bound in the presence of ^{125}I-hCG alone) m=slope, x=log hormone conc. and C=intercept.
ED_{50}^* is the amount of hCG (mIU/tube) required for $B/B_0 = 0.5$.
hCG standards were prepared in 0.05 M phosphate buffer, pH 7.4 containing 0.1% bovine serum albumin and 0.1% sodium azide; undiluted urine and serum (diluted 1:10 in phosphate buffer) obtained from a healthy male.
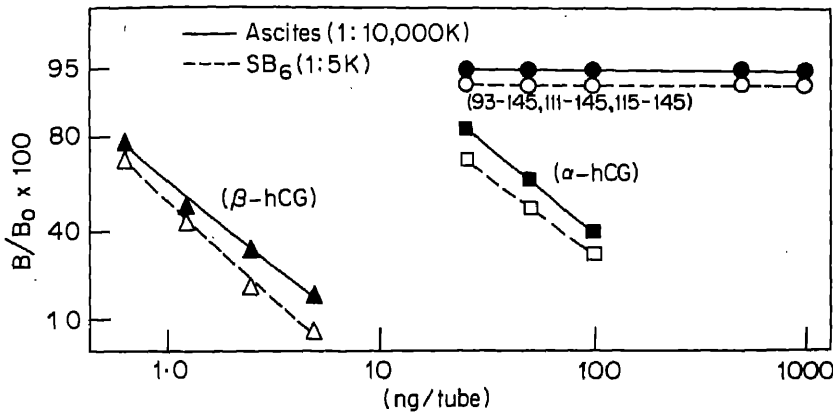
Figure 2. Radioactivity of monoclonal anti-hCG antibody (P_3W_{80}) ascites fluid and SB_6 with alpha and beta subunits of hCG and three carboxy terminal peptides of β-hCG of 93-145, 111-145 and 115-145 amino acid.
β-hCG (SB_6), (△); α-hCG (SB_6), (□); carboxy terminal peptides (SB_6), (O); β-hCG (P_3W_{80}), (▲); α-hCG (P_3W_{80}); (■); carboxys terminal peptides (P_3W_{80}), (●).

howed a similar reactivity. None of the carboxy terminal peptides of βhCG (93-45, 111-145, 115-145) competed with ^{125}I-hCG for binding sites to this monoclonal or B_6 (figure 2). However, SB_6 at lower dilution (1:100) was shown to bind iodinated arboxy terminal peptides (93-145) which was not the case with monoclonals even when tested at 1:100 dilution (unpublished data). The slope of inhibition of SB_6 ith β-hCG was also different from those of monoclonal (figure 2).

hLH at the maximum surge level (Shelly *et al.*, 1973) concentration of 1.2 ng
mIU/tube) per assay tube did not compete for binding of labelled hCG with r
clonal.   60 mIU of hLH/tube was required to obtain a significant cross-react
this assay system (B/B$_0$ =70%) with monoclonals; the amount of hLH prod
similar inhibition was 45 mIU with SB$_6$ (figure 1).   The cross-reactivity of the
body with hLH in the Leydig cell bioassay system is given in table 2.   PRI
devoid of cross-reaction with monoclonal and SB$_6$ upto 10 mIU/ml tested.   D
non-availability of highly purified hFSH and hTSH (free of hLH), the cross-rea
of monoclonals with these hormones has not been tested.

Table 2.   Effect of P$_3$W$_{80}$ antibody on hCG/hLH induced steroidogenesis by Leydig cell

Dilution of P$_3$W$_{80}$ ascites fluid	Percent decrease in testosterone production	
	hCG	hLH
1:100,000	83.6	5.7
1: 10,000	100	5.8
1: 1,000	100	7.3
1: 100	100	24.8

hCG (400 μIU), 2.5 ng of hLH (LER-960) dissolved in 100 μl of phosphate buffer (pH 7.4) wer
preincubated with 100 μl of the indicated dilution of the ascites fluid for 2 h at 37°C followed b
18 h at 4°C.Leydig cell suspension was added to the preincubated hormones and the testoster
one produced was estimated by radioimmunoassay as described in Methods.

Biological neutralization studies

The ability of monoclonal antibodies (P$_3$W$_{80}$) to block the biological activity
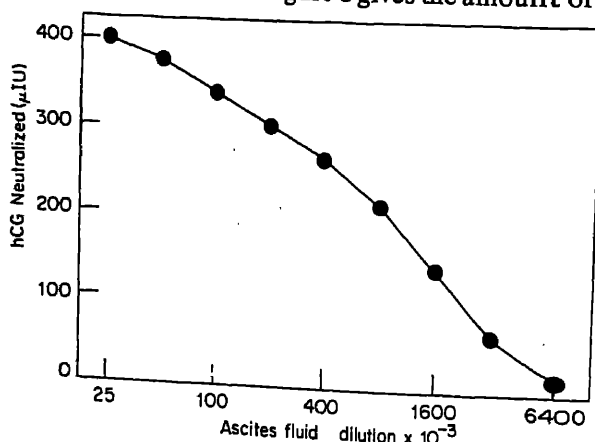hCG was tested *in vitro* and *in vivo*.   Figure 3 gives the amount of hCG neutrali

Figure 3.   Neutralization of the biological activity of hCG as a function of dilution of
monoclonal anti-hCG antibody (P$_3$W$_{80}$) ascites fluid in mouse Leydig cell bio-assay system.
384 μIU of hCG was used and each point represents a mean of quadruplicate determinations.

by various dilutions of ascites fluid in Leydig cell assay system. At 25,000 dilution, the ascites fluid neutralized the entire hCG (384 μIU) taken for the assay: 50% of the hormone was neutralizable at 8×10^5 dilution.

In the mouse uterine weight gain assay, immature mice injected with 0.5 IU of hCG alone showed about three fold increase (5.5 to 15.5 mg) in the wet weight of the uterus as compared to saline injected group. Ascites fluid (upto 1:1000 dilution) given with same quantity of hCG inhibited completely the hormone-induced increase of uterine weight (figure 4).
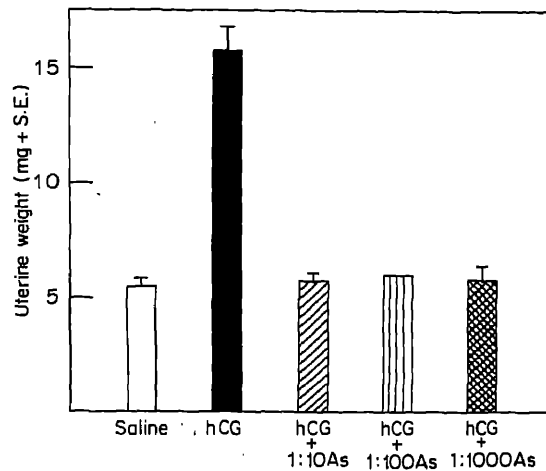
Figure 4. Effect of monoclonal anti-hCG antibody (P_3W_{80}) ascites fluid on hCG induced increase in mouse uterine weight. The bars represent mean values of 6 animals + S.E.

Characterization of heavy and light chains of the monoclonal antibody (P_3W_{80})

The culture fluid of the clone P_3W_{80} as well as the ascites fluid developed in the mouse peritoneal cavity was tested for the light and heavy chain classes. Monospecific antisera against mouse μ, G_1, G_{2a}, G_{2b}, G_3, k and λ chains were utilized. The reactivity was assayed by enzyme linked immunoassay. The

Table 3. Enzyme linked assay for light and heavy chains in the product of the clone P_3W_{80}

Test product	Test antiserum							
	Blank	μ	G_1	G_{2a}	G_{2b}	G_3	K	λ
	Absorbance at 440 nM							
Culture fluid from NSI myeloma cells	—	0.048	0.048	0.041	0.041	0.051	0.047	0.023
P_3W_{80} culture fluid	0.11	0.18	2.64	0.14	0.08	0.095	1.395	0.055
P_3W_{80} mouse ascites fluid	0.129	0.304	2.66	0.031	0.105	0.080	1.358	0.057

The assay was carried out as described in Methods.

product of the P_3W_{80} clone in culture or from ascites fluid reacted only with
mouse G_1 and kappa serum as evidenced by absorbance at 440 nm which is sh
in table 3. Other wells showed negligible absorbance. Culture fluid obta
from myeloma cells (NSI) gave negligible absorbance with all the tested antis

Discussion

The antibody made by P_3W_{80} clone is an IgG_1, k type; it can bind the compler
and can bring about the antibody induced lysis of cells bearing hCG. This ma
an interesting trait, as anti-hCG antibodies have been observed to exerci
complement-dependent cytotoxic action against choriocarcinoma cells *in*
(Currie, 1967; Talwar, 1980).

The antibodies are of neutralizing type. The antibodies abrogate the
activity of hCG both *in vitro* and *in vivo.* It may be recalled that antibc
generated against carboxy terminal peptides unique to β-hCG fail to intercep
hCG action *in vivo* (Louvet *et al.,* 1974; Matsuura *et al.,* 1976). This may be pa
due to the fact that antibodies generated by carboxy terminal peptides
comparatively of low affinity with K_a of 1.2×10^9 L/M (Chen *et al.,* 1980).
however, also possible that the epitopes against which the antibodies are dire
has importance. The antibodies against carboxy terminal peptides read seque
located in a tetrapeptide and a dipeptide sequence, whereas the antibo
produced by this clone bind to other epitopes or conformation in the hCG/β-h
molecules. These antibodies are devoid of recognition of carboxy term
peptides of hCG. Leydig cell receptors do not respond to these peptides and
determinant(s) inducing biological effect of the hormone reside in core part
hCG and still better in the associated hCG molecule (Ramakrishnan *et al.,* 19
Thus the binding characteristics of the monoclonal antibodies are of interest
amenable to applications. Preliminary studies in our laboratory demonstrate
ability of these antibodies to terminate pregnancy in mouse.

These antibodies just as other hybrid cell clone products can be obtaine
extremely high titres. The present antibody binds 30 to 40% of ^{125}I-hCG at
dilution. The supply of these antibodies is abundant and theoretically unlimit
These can be used for radioimmunoassays and enzyme linked assays;
specificity is very similar to SB_6. These antibodies can also be used for neutral
tion of the bioactivity of hCG.

Acknowledgements

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Analysis of bovidae genomes: Arrangement of repeated and single copy DNA sequences in bovine, goat and sheep

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Abstract. Approximately 43-60% of the total genome in bovine, goat and sheep consisted of interspersed repeated and single copy DNA sequences. Most of the interspersed repeated DNA sequences were 1500-2400 nucleotide pair long while a minor portion was more than 4000 nucleotide pair long in goat and sheep and 3200 nucleotide pair long in bovine. About 1/3rd of single copy sequence were interspersed and their length was in the range of 1000-1500 nucleotide pairs.

Introduction

In the family Bovidae, extensive studies were carried out on satellite DNAs in bovine, goat and sheep (Fillipski *et al.,* 1973; Curtain *et al.,* 1973; Votavova and Sponar, 1974, 1975a, 1975b; Votavova *et al.,* 1975; Kopecka *et al.,* 1978; Forstova *et al.,* 1979). Repeated DNA sequences were also characterized in these three species (Britten and Kohne, 1968, Voltavova *et al.,* 1972, 1973; Mehra *et al.,* 1980). In buffalo, another member of the family bovidae, repeated DNA sequences were identified (Mehra and Ranjekar, 1979) and their arrangement with respect to single copy DNA sequences was described (Mehra and Ranjekar, 1981). Recently, a report on the sequence organization of bovine DNA has appeared (Mayfield *et al.,* 1980).

Our interest in studying the genome organization in bovine, goat and sheep was to assess the similarity, if any, in the mode of DNA sequence arrangement of repeated and single copy DNA sequences. We also wished to compare the genome organization of these three species with the available data in buffalo and other mammalian species.

Materials and methods

The experimental details about DNA isolation and fragmentation, DNA reassociation kinetics, (Ranjekar and Murthy, 1973; Ranjekar *et al.,* 1974) thermal denaturation of DNA, (Ranjekar *et al.,* 1976) determination of S_1 nuclease resistance of DNA duplexes and sizing of DNA by agarose gel electrophoresis have been described (Seshadri and Ranjekar, 1980).

Results

Reassociation of DNAs of increasing fragment lengths

From the reassociation kinetic studies of the sonicated DNAs (550 nucle﬊᷄
pairs), the approximate proportion of repetitive DNA was calculated to be: 4ᷓᷓ
bovine; 28% in goat; and 26% in sheep (Mehra *et al.*, 1980). Reassociation ᷛ᷍ ᷓ
DNAs of bovine, goat and sheep of fragment lengths upto 6190 nucleotide ᷛ᷀᷍
were studied in the Cot range of 10^{-1} to 100. DNA sequences forming duplexesᷛᷛ
Cot 50 in bovine and sheep and Cot 1 in goat were assumed to be mainly repetᷛᷛᷓᷓ
It is apparent from figure 1, the proportion of DNA binding to hydroxyapᷛᷛᷓᷓ
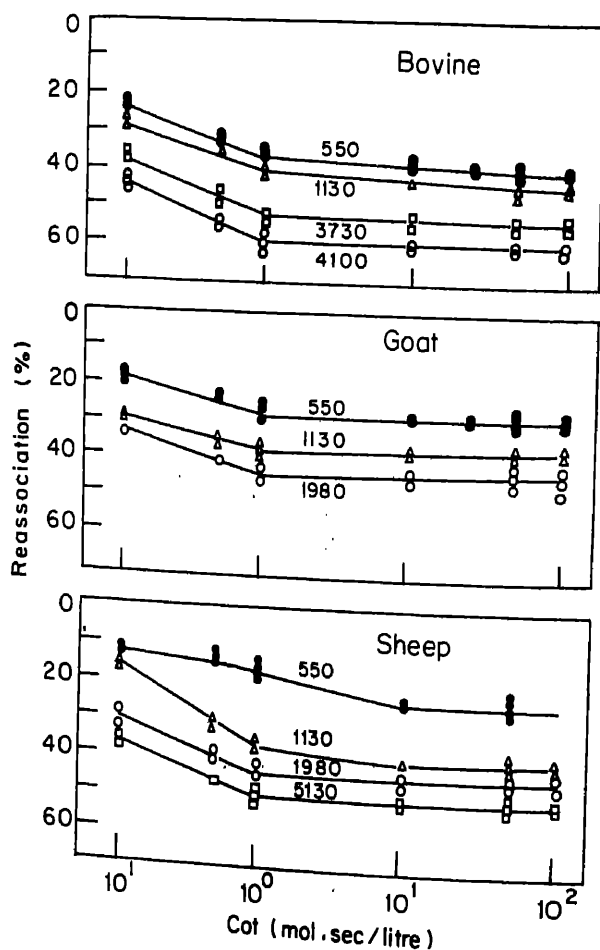
Figure 1. Hydroxyapatite reassociation kinetics of DNAs of different fragment lengths in 0.12
M sodium phosphate buffer, pH 6.8. The DNA fragment length is indicated on each curve as
nucleotide pairs (np). DNA concentrations ranged from 25 to 200 µg/ml. Approximately 500-
600 µg of DNA was used for each experiment. The DNA was first denatured at 100°C for 10
min and was immediately incubated at 62°C to obtain a desired Cot value. Separation of single
stranded and double stranded DNA was carried out by hydroxyapatite column chromatography.
The numbers indicate the length of nucleotide pairs.

increased with the DNA fragment length in all the three species. For example, the increase was from 40 to 60% in bovine, 24 to 43% in goat and 26 to 50% in sheep It could also be noticed from figure 1 that there was either a very slight increase or no increase in the DNA fragment length. An increase in binding of DNA to hydroxy-apatite with the increase in the DNA fragment length could be due to interspersion of repeated and single copy DNA sequences or due to fragment length alone (Davidson *et al.*, 1973; Graham *et al.*, 1974; Wetmur and Davidson, 1968).

However, the latter possibility can be ruled out from table 1, where it is seen that the observed K values are several times higher than the predicted values indicating

Table 1. Comparison of the experimental and the predicted rate constants (K) of the repeated DNA fractions of different framgent lengths.

DNA	Frament length (np)	Cot 1/2	Observed[a] K	Predicted[b] K	K observed / K predicted
Bovine	550	4×10^{-2}	25	—	—
	1130	2.3×10^{-3}	434.78	36.23	12
	4100	2×10^{-3}	500	69.44	7.2
Goat	550	10^{-2}	100	—	—
	1130	3.4×10^{-4}	2857	144.9	19.7
	1980	10^{-4}	2500	192.3	13.00

[a]$$K = \frac{1}{Cot\ \frac{1}{2}}$$

[b]Calculated using $\frac{K1}{K2} = \left[\frac{(L1)}{(L2)}\right]^{0.5}$ where K1 and K2 are the rate constants for the reassociation of short (L1) and long (L2) fragments respectively (Wetmur and Davidson,1968).

thereby the presence of interspersion of repeated and single copy DNA sequences in bovine, goat and sheep. The proportion of total DNA consisting of interspersed DNA sequences was 60% in bovine, 43% in goat and 50% in sheep. From figure 1 it is evident that in all the three species, there is a substantial increase in the extent of binding of DNA to hydroxyapatite at Cot 0.1; for example, in bovine, 23% and 43% of 550 and 4100 nucleotide pair long DNA respectively form duplexes, whereas in sheep, 12% of 550 nucleotide pair long DNA and 37% of 5130 nucleotide pair long DNA reassociate. These data indicated that in addition to the interspersion of repeated and single copy DNA sequences, there was interspersion among repeated DNA sequences themselves.

Hyperchromicity of repetitive DNA fractions of different fragment lengths

Hyperchromicity measurements of repeated DNA sequences of different fragment size were carried out to estimate the duplex content or the fraction of nucleotides paired. Cot 50 fraction in bovine and sheep and Cot 1 fraction in goat were isolated from DNAs of varying fragment size. As shown in table 2, the T_m values

Table 2. Thermal denaturation analysis of reassociated repetitive duplexes isolated from DNAs of different fragment lengths.

DNA	Frament[a] length (np)	Fraction[b] length (np)	Hyperchromicity[c]	Tm^d	$Q \times H^e$ Native(H)	Fraction of duplex From[f] chromicity studies	From $S_1{}^g$ nuclease data	Average[h] length of duplex per fragment
1	2	3	4	5	6	7	8	9
Bovine	550	0.40±0.01 (7)	0.13 ±0.01 (8)	74.0±0.32 (7)	0.208	0.507		279
	1130	0.44±0.08 (3)	0.088±0.007 (4)	71.0±0.11 (4)	0.155	0.294		332
	3730	0.54±0.03 (3)	0.076±0.002 (4)	72.0±0.08 (4)	0.164	0.233		869
	4100	0.60±0.02 (5)	0.08 ±0.004 (6)	70.0±0.25 (3)	0.192	0.253	0.257±0.03	1037
Goat	550	0.28±0.090 (7)	0.18 ±0.02 (5)	73.0±0.2 (8)	0.188	0.755	(3)	415
	1130	0.37±0.074 (5)	0.15 ±0.02 (5)	73.5±0.13 (5)	0.214	0.614		694
	1980	0.43±0.084 (5)	0.122±0.03 (6)	74.0±0.29 (6)	0.203	0.483	—	956
Sheep	550	0.26±0.04 (5)	0.172±0.029 (6)	73.0±0.23 (6)	0.167	0.683		376
	1130	0.40±0.02 (6)	0.156±0.01 (6)	74.0±0.13 (3)	0.233	0.612		692
	1980	0.45±0.04 (6)	0.144±0.04 (5)	75.0±0.18 (6)	0.242	0.559		1107
	5130	0.50±0.05 (6)	0.077±0.04 (6)	69.4±0.52 (6)	0.144	0.262	0.216±0.02 (3)	1344

[a] Fragment length was measured using neutral sucrose density centrifugation.

[b] DNA sample was denatured and incubated to Cot 50 in bovine and sheep and Cot 1 in goat and passed over hydroxyapatite at 62°C in 0.12 M sodium phosphate buffer, pH 6.8.

[c] Hyperchromicity was determined by melting the duplex in 0.12 M sodium phosphate buffer (pH 6.8) in a Gilford Spectrophotomer. Hyperchromicity was calculated as follows:

$$H = \frac{A_{260}(98°C) - A_{260}(52°C)}{A_{260}(98°C)}$$

[d] The temperature at which half of the DNA was melted

[e] Fraction of bases in the repetitive region $= \dfrac{\text{Fraction bound} \times \text{observed hypercromicity}}{\text{Native DNA hyperchromicity}}$

[f] $D = \dfrac{\text{Hyperchromicity of Cot fraction} \quad - \text{Hyperchromicity due to single strand collapse}^*}{\substack{\text{Hyperchromicity of duplex} \quad - \text{Hypercromicity due to single strand collapse}^{**} \\ \text{regions as obtained by S1} \\ \text{nuclease studies}^{**}}}$

   * Hyperchromicity due to single strand collapse is 2.9%, 1.87% and 1.78% for bovine goat and sheep respectively.

   ** Hyperchromicities of S1 nuclease resistant duplex regions are obtained as described in the text and the values are given in Table 3.

[g] Calculated Using

Duplex Content $= \dfrac{\text{S1 nuclease resistance}}{\text{Fraction bound to hydroxyapatite at the fragment length measured}}$

[h] Average length of duplex per fragment—Fraction of the duplex×fragment length. The number in parenthesis indicates the number of experiments carried out.

of repetitive DNA duplexes of different size in each species exhibited only a slight variation in the range of 0.5-1.5°C indicating that similar sequences were present on DNA strands of different fragment lengths. The hyperchromicity values, however, showed a marked variation (figure 2). There was a progressive decrease from 13% to 8% in bovine, 18% to 12% in goat, 17% to 7% in sheep in hyperchromicity with increase in the DNA fragment length. A most likely explanation for such decrease in the hyperchromicity with increase in the DNA fragment size was the presence of increasing amounts of unreassociated single stranded regions along the reassociated duplexes. These data thus provide another evidence for the presence of interspersion i.e. occurrence of repeated and single copy DNA sequences on the same DNA strand. The average size of the interspersed repetitive sequences computed from the hyperchromicity measurements was in the range of 1040-1340 nucleotide pairs in all the three species (table 2).
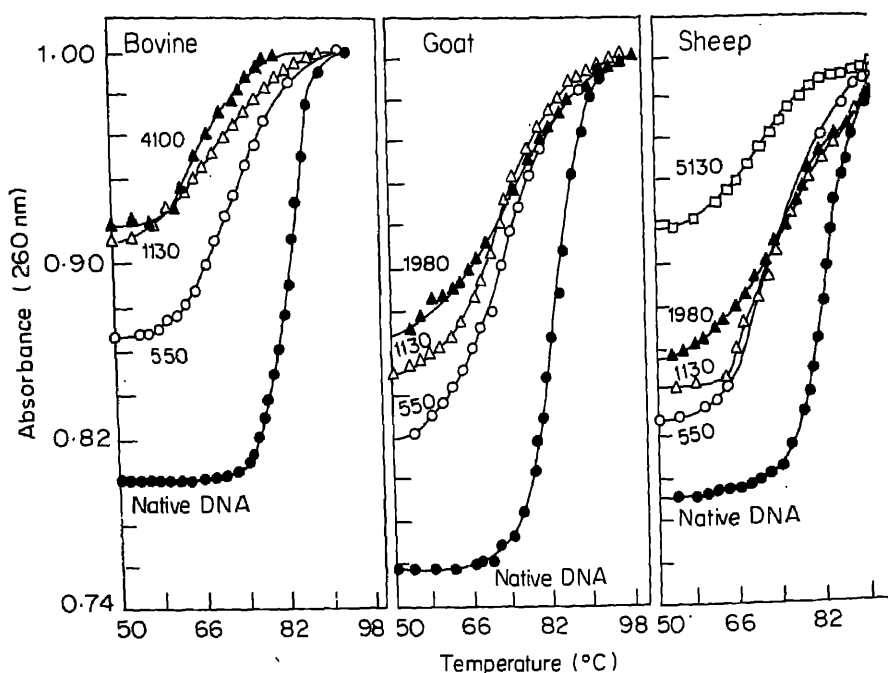
Figure 2. Melting profiles of Cot 50 fractions of bovine and sheep and Cot 1 fractions of g(
of different fragment lengths. Gilford spectrophotometer (Model 250) with thermoprogramn
(Model 2527) and analog multiplexer (Model 6046) was used for these measurements. T
DNA solution (25-50 μg/ml) in 0.12 M sodium phosphate buffer, pH 6.8 was heated at a rate
1°C/min and the changes in absorbance at 260 nm were recorded. *Escherchia coli* DNA w
used as standard. The numbers indicate the length of nucleotide pairs.

S1 nuclease resistance of repetitive DNA duplexes

Cot 50 fraction of bovine and sheep DNAs of fragment lengths 4100 and
nucleotide pairs, respectively were treated with single strand specific S1 nucl(
The S1 nuclease resistant duplexes were isolated by hydroxyapatite col
chromatography. The hyperchromicity measurements of the DNA fractions (
carried out before as well as after S1 nuclease treatment. S1 nuclease resis
DNA showed a substantial increase in the hyperchromicity (table 3). Howevei
significant changes were observed in the Tm values of the DNA fractions be
and after S1 nuclease treatment.

In bovine, 26% of the 4100 nucleotide pair DNA was S1 nuclease resistant
hence in duplex form. This estimate compares well with the duplex cont
estimated from the melting data (table 2). In sheep, 21.6% of the 5130 nucleot
pair DNA was S1 nuclease resistant. This value was rather low when compare(
that of 26% from the hyperchromicity measurements (table 2).

Table 3. Hyperchromicity of S1 resistant repetitive duplexes.

	Hyperchromicity	
	Before S1 nuclease treatment	After S1 nuclease treatment
Bovine Cot 50 DNA (4100 np)	8.0±0.4	22.7±0.5
Goat Cot 1 DNA (1980 np)	12.2±0.3	23.2±0.7
Sheep Cot 50 DNA (5130 np)	7.7±0.4	24.3±0.4

Sizing of repetitive DNA

A direct estimate of the size of repetitive DNA sequences was obtained by analyzing S1 nuclease resistant repetitive duplexes using agarose gel electrophoresis. Agarose gel scans showing the banding patterns of the S1 nuclease resistant Cot 50 fractions in bovine and sheep and Cot 1 fraction in goat are shown in figure 3. One main sharp peak and one rather small peak were observed in all the DNA fractions. The length of the repetitive DNA in the sharp peak was estimated to be in the range of 1500-1650 nucleotide pairs in bovine, 2100-2400 nucleotide pairs in goat and 1500-2000 nucleotide pairs in sheep whereas that in the small peak was 3200 nucleotide pairs in bovine and 4000-4400 nucleotide pairs in goat and sheep.

Sizing of interspersed single copy DNA sequences

To arrive at an estimate of the length of the interspersed single copy DNA sequences, a curve relating to the fraction of DNA fragment binding to hydroxyapatite was plotted against the DNA fragment length. There was a linear relationship between DNA fragment length and per cent binding to hydroxyapatite till the DNA fragment length equalled the average distance between repeated sequences. The position of the change in slope, therefore, was interpreted as the approximate length of single copy sequences.

From figure 4, it is observed that two slopes are present in all the species and the change in slope occurs at a fragment length of 1000-1250 nucleotide pairs in bovine, 1250-1500 nucleotide pairs in goat and 1000-1350 nucleotide pairs in sheep. Thus, the length of the single copy DNA sequences vary in the range of 1000-1500 nucleotide pairs in all the three species.
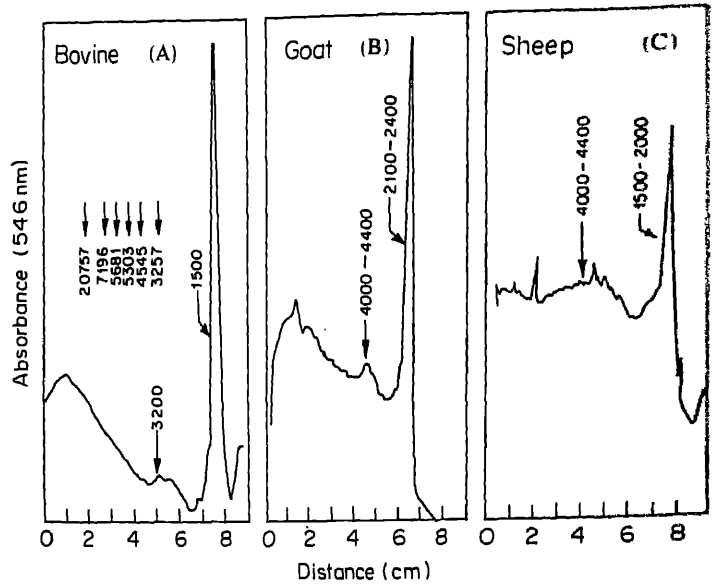
Figure 3. Agarose gel electrophoresis of S1 nuclease resistant repetitive DNA duplex

(A) Bovine Cot 50 DNA; (B) Goat Cot 1 DNA; (C) Sheep Cot 50 DNA.

A standard graph of log molecular weight versus distance travelled in cms was first using the electrophoretic data of ECORI treated lamda DNA digest. Six peaks were of and their molecular weight values were taken from Thomas and Davis (1975). For e phoresis, 1.4% agarose in Tris/borate buffer (0.089 M Tris, 0.089 M boric acid, pH 8.5 mM Na$_2$ EDTA) was used. About 2-3 μg of DNA in 0.12 M sodium phosphate buffer, adjusted to 10% sucrose was layered under the buffer on the top of each gel and electre esis was carried out at 50 V for 5 h at room temperature. The gels were stained with t toluidine blue (Philippsen and Zachau, 1972), destained with distilled water and scani Gilford spectrophotometer using a gel scanner accessary at 546 nm.
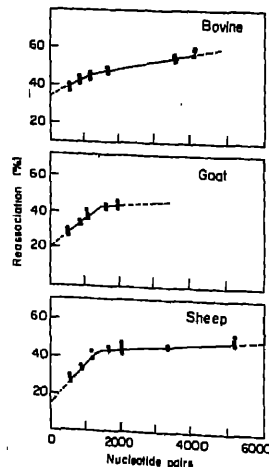
Figure 4. Per cent binding of DNA to hydroxyapatite at Cot 50 in bovine and sheep and Co in goat as a function of fragment length. The experimental details are described in the lege of figure 1.

Discussion

The present study has enabled us to draw the following conclusions:

1. There is an interspersion of repeated and single copy DNA sequences in bovine, goat and sheep.

2. There is also an interspersion of highly repetitive DNA (sequences reannealing by Cot 0.1) and intermediately repetitive DNA (sequences forming duplexes in the Cot range of 0.1 to 50 in bovine and sheep and 0.1 to 1.0 in goat).

3. A major portion of the repeated sequences is 1500-2400 nucleotide pairs long and a minor portion is 3200-4400 nucleotide pairs long.

4. The repeated sequences are intervened by single copy sequences of 1000-1500 nucleotide pairs. Approximately 43-60% of the genome consists of such interspersed sequences. The pattern of DNA sequence organization in the present three species is grossly similar among themselves and is also comparable to that in buffalo (Mehra and Ranjekar, 1981). However, it is different from that observed in other mammalian species such as mouse (Cech and Hearst, 1976; Ginelli *et al.*, 1977), rat (Bonner *et al.*, 1973; Wu *et al.*, 1977) and human (Deninger and Schmid, 1976; Ginelli and Corneo, 1976). Bovine, buffalo, goat and sheep belong to the family Bovidae. The chief mechanism of karyotype evolution in this family is Robertsonian fission or fusion (Robertson 1916; Wurster and Benirschke, 1968). More recent studies employing chromosomal banding techniques have shown strikingly similar banding patterns in the chromosomal arms of calf, sheep and goat and nearly exact structural homologues throughout the chromosome. A similarity in DNA sequence organization in these three species is in keeping with the cytological data.

A substantial proportion of repeated sequences in bovine, goat and sheep represents satellite DNA sequences (Curtain *et al.*, 1973; Makaya *et al.*, 1978; Forstova *et al.*, 1979). Using restriction endonucleases, Mowbray *et al.* (1975) and Philippsen *et al.* (1975) have shown that in bovine satellite DNA, fragments of 1300 nucleotides are alternating with 1550 nucleotide long fragments. According to Botchan (1974) the repeat unit of bovine satellite I DNA is 760 base pairs in length. Recently, Mayfield *et al.* (1980) have shown that 6 to 10% of the total bovine DNA average 350 nucleotides in length. In the present work, we have determined the fragment size of all the DNA sequences forming duplexes by Cot 50 in bovine and sheep and Cot 1.0 in goat. Our size estimates of the interspersed repeated DNA sequences are, therefore, average values in all the three species.

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ERRATA

Dopaminergic mediation of Y -aminobutyric acid in the control of prolactin release: Plasma prolactin and brain tyrosine hydroxylase levels in ovariectomized conscious rats

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Abstract. A novel polycationic ionen was synthesized and fractionated on carboxymethyl-Sephadex using a salt gradient in 7M urea. A series of oligomers of discrete length were characterised by ultraviolet spectra. The ultraviolet spectra of oligomers revealed a new band centred at 232.5 nm which was probably due to exciton splitting. Thermal denaturation studies indicated both stabilization of the helix conformation and a higher degree of cooperativity in the melting of DNA (oligomers)$_n$ complex as compared to native calf thymus DNA. Ionen oligomers exhibited large extrinsic Cotton effect at 232.5 nm which could be attributed to exciton interaction.

Keywords. Ionen-oligomers; DNA; melting temperature; circular dichroism; exciton splitting.

Introduction

It is well known that various types of polycationic ionens interact with nucleic acids which are polyanions (Mulimani *et al.*, 1977; Day *et al.*, 1978; Mulimani and Day, 1980, 1981). Polycationic ionen was synthesized and its interaction studied with DNA. Ionen exhibited an extrinsic Cotton effect attributable to the ordered arrangement of the aromatic chromophore along the DNA helix (Bhat *et al.*, 1977). The interaction between DNA and ionen polymers was examined to determine the binding of cationic polymers with DNA dependent on the charge density of the polycation (Mita *et al.*, 1977). Interaction of oligopeptides with DNA showed that the peptides which contain aromatic amino acids at the C terminus markedly decreased the specific visocity of DNA. Based on these as well as proton magnetic resonance studies of oligopeptide-DNA complexes, "nonclassical intercalation" was proposed (Gabbay, 1978) whereby the aromatic residues of oligopeptides were partially inserted between base pairs of DNA, leading to bending of the helix at the point of intercalation.

This paper describes the synthesis, fractionation and ultraviolet (uv) characterization of polycationic ionen and the interaction of ionen oligomers with DNA by thermal denaturation and circular dichroism (CD).

Abbreviations used: uv, ultraviolet; CD, circular dichroism.

Materials and methods

Synthesis of p-nitrophenyl blocked polymer

Dimethyl *p*-nitrobenzylamino bromide methylene *p*-phenylmethylene dimeth
amine bromide *p*-nitrotoluene was made by blocking the ends of polymer with
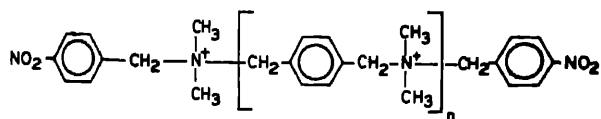
chromophore that absorbs light at longer wavelengths than the polymer, so that t
length could be determined from the absorption spectrum. The ionen w
synthesized according to the procedure of Rembaum *et al.*, (1967, 1968
Dimethylamine passed through a solution of α-bromo *p*-nitrotoluene (0.01 mol) a
0.01 mol of α, α'-dibromo *p*-xylene were dissolved in approximately 50 ml
dimethylformamide. The pale yellow precipitate which appeared almost imm
diately was filtered, washed with dimethyl formamide and acetone and dried. T
product was characterised by ultraviolet and nuclear magnetic resonance (nmr

Polycationic ionen (170 mg) was applied to CM Sephadex column (4.3×3.5 cm
equilibrated with 0.3 M NaCl and eluted with a gradient of 0.3 M to 0.8 M Na
(figure 1). Peak fractions in the elution profile were characterised by their u
spectra (figure 2 and 3). It is clearly evident from a comparison of the uv spectra
oligomer fractions that the absorbance at 220 nm was due to phenyle
chromophore. A new band centered at 230.5 nm appeared in the case of oligome
(n>1) and increased in relative intensity with each successive oligomer fractic
and became a sharp spike at n>3 (figure 3). The absorbance at 220 nm divided l
the absorbance at 263 nm gave the proportional relative numbers (n) of phenyle
and *p*-nitrophenyl groups in the oligomer fractions.

DNA was purchased from Sigma Chemical Co., St. Louis, Missouri, USA as i
sodium salt (sodium deoxynucleotide or calf thymus DNA highly polymerised
Sodium deoxynucleate (1 mg/ml) was added to sodium chloride 0.01 M and sodiu
citrate 0.001 M pH 7 buffer) over a few drops of carbon tetrachloride hereaft
referred to as buffer A. DNA was estimated spectrophotometrically assumin
$e = 6.6 \times 10^3 cm^{-1} M^{-1}$ at 260 nm. The A_{260}/A_{330} ratio of this solution was 2.3. Th
components of the reaction mixture were added in the following order: DN
(7.5×10^{-5}), sodium chloride (1 M), EDTA (0.001 M) and ionen oligomer. Th
total volume of the mixture was made upto 4 ml by diluting with SSC buffer A. Th
ionen oligomer and DNA ratio is represented by r. Before determining the meltin
temperature (T_m) the uv absorbance of the mixture was measured using a Gilfor
spectrophotometer model 240. Thermal denaturation studies were carried out i
microcuvettes using a Gilford spectrophotometer equipped with thermo
programmer, model 2527. The samples were heated from room temperatur
to 100°C at a rate of 1°C/min. No correction for thermal expansion was made to th
absorbance readings in routine experiments. CD spectra of DNA-ionen oligomer
at different r in buffer A was obtained using Cary 60 spectrophotometer with mode
6002 CD attachment.

Results and discussions

Separation of oligomers with polycationic ionen by CM sephadex was complete for all oligomers (figure 1). It is evident from the elution profile that nine main peaks
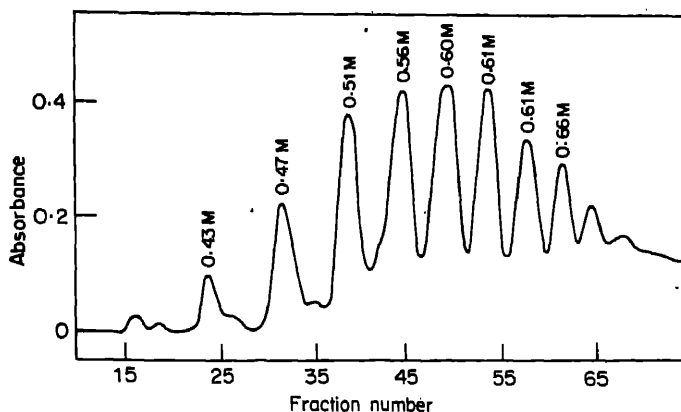

Figure 1. Separation of oligomers from ionen on CM-Sephadex with salt gradient. Numbers above the peaks are the concentrations of sodium chloride.

were well separated and two were not. The uv spectra of the oligomers revealed a new peak at 232.5 nm that did not appear in the spectrum of monomers (figures 2 and 3). The melting profiles of the complexes formed between DNA and oligomer (n=7) at different r are shown in figure 4.(Oligomers)$_n$ contribute towards raising the T_m values of all molecules or every portion of DNA. The melting band at
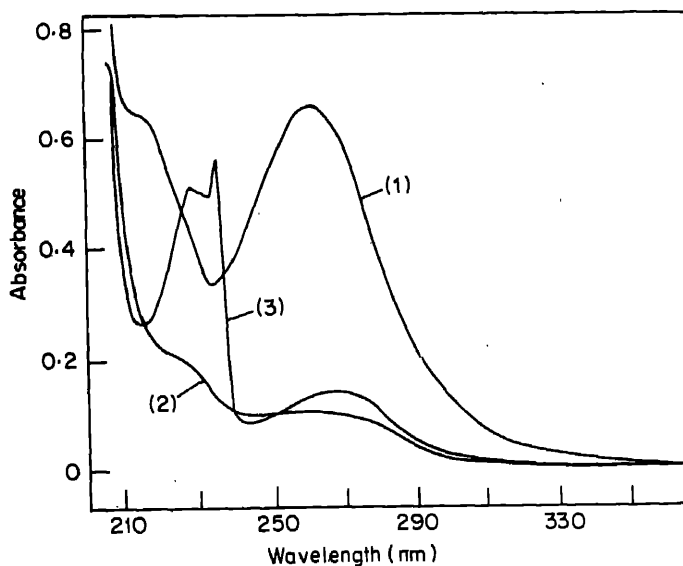
Figure 2. 1) UV spectrum of fraction no 25 (n=1).
2) UV spectrum of fraction no 34 (n=2).
3) UV spectrum of fraction no 49 (n=3).

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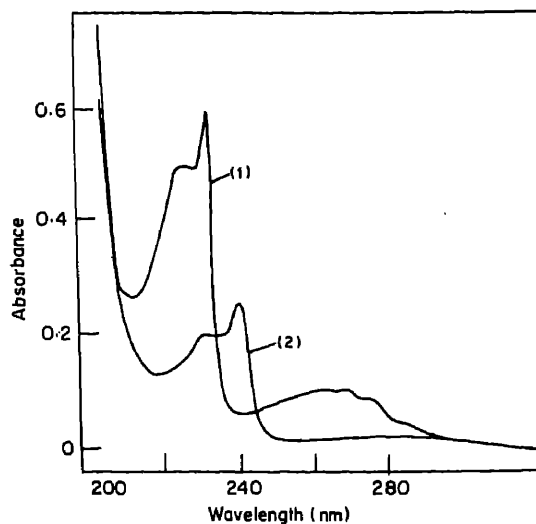
Figure 3. 1) UV spectrum of fraction no 54 (n=4)
2) UV spectrum of fraction no 63 (n=7).
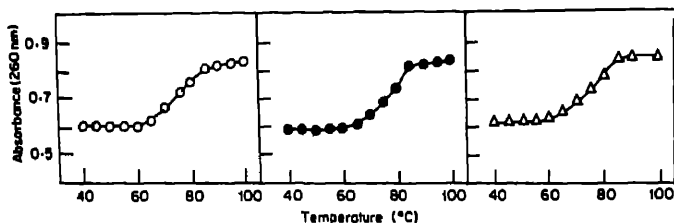
Figure 4. Melting profile of DNA oligomer n=7.
O=0.05, ●=0.1, Δ=0.2.

$70°C$ (T_m) corresponds to the melting of native DNA. The melting band appears at $75°C$ when oligomer (n=7) is added at the different ratio. Addition of oligomers (n=4 and 7) to DNA gave an extrinsic band at 232.5 nm and lowered the positive lobe centred at 275 nm until at higher r values it became slightly negative (figures 5 and 6).
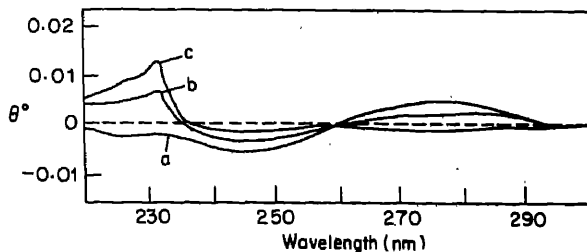
Figure 5. CD spectra of oligomer n=4 with calf thymus DNA.
a) DNA; b) r=0.21; c) r=0.35.
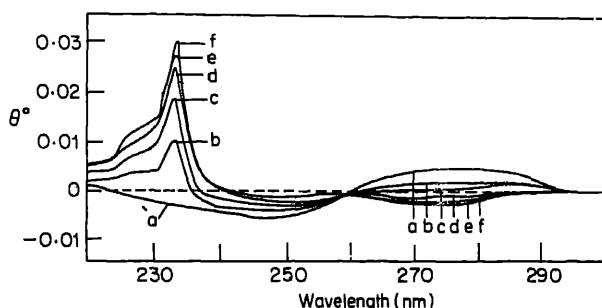
Figure 6. CD spectra n=7 with calf thymus DNA.
a) DNA; b) r=0.077; c) r=0.144; d) r=0.216; e) r=0.288; f) r=0.36.

The uv spectra of oligomers revealed a new band centred at 232.5 nm that did not appear in the spectrum of monomers. This can be explained by the phenomenon of exciton splitting due to resonance interactions between excited states of weakly coupled aggregate systems (Kasha 1963). Interaction between the transition dipoles of identical unit molecules in a regular array yields discrete exciton states,only a very few of which can be reached by allowed transitions. This gives rise to striking effects in the absorption spectrum of aggregates as compared to a unit molecule such as band splitting or shifts and hyper or hypochromism. This concept has been applied to molecular crystals dye aggregates in solution as well as polymers (Kasha 1963, Davydov 1962, Levison *et al.,* 1957). Exciton splitting was predicted for helical polypeptides and it was subsequently shown that α helix is hypochromic as compared to the random coil and exhibits a shoulder to the side of the peptide band absorbance at 190 nm (McRae and Kasha 1958, Moffitt 1956). The uv spectra of nucleic acids have also been analyzed in terms of exciton splitting. According to generalised selection rules for helical polymer the light absorption due to the component of the transition dipoles of the interacting chromophores polarised parallel to the helix axis will be red shifted in comparison to monomer (Kasha 1963). The new band at 230.5 nm and its shoulder must arise from transitions of the phenylene chromophore below 210 nm. The oligomers and polymer would be expected to maintain a regular conformation in solution due to intramolecular hydrophobic interactions of the aromatic groups.

The melting band at 70°C represents the melting of free base pairs of DNA not bound by oligomers. The melting temperature of the free base pair increases after addition of oligomers. Thus thermal denaturation indicated both stabilization of the helix conformation and higher degree of cooperativity in the melting of DNA—(oligomer)$_n$ complex as compared to native calf thymus DNA. The data presented here are consistent with the hypothesis (Gabbay, 1978) which lead to an understanding of various aspects of DNA-polycation interactions as reflected in melting profiles. Previous reports of thermal denaturation of (Arg)$_n$ (Kawashima *et al.,* 1969) and (Ornithine)$_n$ (Kawashima and Toshio, 1978) with DNA complexes also have noted an increase of T_m of free base pairs when basic oligomers were added to DNA. The large extrinsic Cotton effect induced by the oligomers at 232.5 nm is attributable to exciton interaction. The chiral interaction of two or

more isolated but spatially close chromophores give rise to Davydove split (Harada *et al.*, 1975). The sign of the first Cotton effect is a consequence of the dihedral angle between the interacting electric dipole vectors. If the angle is less than 180°C (in a clockwise sense) the sign of the first Cotton effect is positive and if it is greater than 180° (clockwise) the sign of the first Cotton effect will be negative. The intensity of the Cotton effect is inversely proportional to the square of the interchromophoric distances if the angle between the chromophores is constant. Change in dihedral angle affects the CD amplitude which is zero at 0° and 180° and for vicinal dibenzoate reaches a maximum at 70°C. The second Cotton effect is quite often minimal or absent which is attributed to the asymmetric pattern of the corresponding electronic absorption band (Harada *et al.*, 1975). The exciton chirality method would predict a positive extrinsic band if the oligomer were aligned in one of the grooves of the polynucleotide and this is exactly what is observed. The free oligomers in solution show no CD signal. They are in helical conformation but without the constraint of the right handed polynucleotide helix the oligomers will exist as a racemic mixture of right and left handed helical conformation.

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Isolation and characterization of lysosomal alpha-mannosidase of placental tissue

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Abstract. The acidic α-mannosidase was purified 4400-fold by affinity chromatography on Concavalin A-Sepharose and heat treatment at 65°C in the presence of 1 mM zinc ion. The enzyme did not resolve into multiple forms as in the case of enzymes from human liver and human kidney. The pH optimum of the enzyme was 4.2 in citrate-phosphate buffer. The K_m value for p-nitrophenyl-α-D-mannose was 1.9 mM. The molecular weight of the enzyme determined by gel filtration was 300,000. The enzyme contained 10.6% neutral sugars.

Keywords. Placental tissue acid;α-mannosidase; Concanavalin A-Sepharose chromatography.

Introduction

Three types of α-D-mannosidase (EC 3.2.1.24) with different localizations were described in mammalian tissues. Acid α-mannosidase with pH optimum of 4.0-4.5, a typical lysosomal acid hydrolase, was widely distributed in mammalian tissues and plant seeds (Snaith and Levvy, 1973). The enzyme, with a pH optimum of 6.0-6.5 was first reported by Suzuki *et al.* (1969) and later purified to homogeneity by Shoup and Touster (1976) from the cytosol of rat liver. A third form of the enzyme with a pH optimum of 5.5,a component of Golgi-membrane of rat liver (Dewald and Touster, 1973), was solubilized by treatment with 0.5% deoxycholate. The enzyme was different from acid and neutral α-mannosidase not only on the basis of pH optimum but also on the basis of its kinetic properties as well as on its electrophoretic mobility. These enzymes did not require any lipid co-factor except for Zn^{2+} for the rat epididymis enzyme(Snaith and Levvy,1968).Forsee and Schutzbach (1981) partially purified after solubilization from rat liver microsomes, an α-mannosidase which required calcium ion as well as phosphatidyl choline or phosphatidyl ethanolamine for its activity. The enzyme was specific for α-1,2-mannosyl-mannose linkage and had a pH optimum between 5.0-5.5.

In the disease, mannosidosis, which occurs in both man (Ockermann, 1967; Kjellman *et al.*, 1969) and cattle (Hocking *et al.*, 1972), lysosomal acid α-D-mannosidase is absent but the neutral form is present in the normal amount (Carroll *et al.*, 1972). In this paper we report the purification and properties of lysosomal acid α-D-mannosidase from human placental tissue an easily available human tissue.

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Materials and methods

p-Nitrophenol,*p*-nitrophenyl-α-D-mannose,*p*-nitrophenyl-α-L-fucose,*p*-nitrophen α-D-galactose, *p*-nitrophenyl-β-D-galactose, *p*-nitrophenyl-α-D-glucose, nitrophenyl-β-2-acetamido-2-deoxyglucose, crystalline bovine serum album bovine serum albumin, acrylamide, N,N'-methylene-*bis*-acrylamide, N,N,N',] tetramethylene diamine, Coomassie Brilliant Blue R, α-methyl-D-glucose a cyanogen bromide were purchased from Sigma Chemical Co., St. Louis, Missou USA. Sephadex G-200, Sephadex G-50, blue-dextran 2000, DEAE-Sephadex A-, and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsal Sweden. The molecular weight marker proteins were purchased from Pier Chemical Co., Rockford, Illinois, USA. All other chemicals used were Analytical Reagent grade. *Canavalia gladiata* and *Canavalia ensiformis* we purchased locally. Concanavalin A-Sepharose 4B affinity column: Concanaval A was isolated from *C. gladiata* or *C. ensiformis* and purified according to tt method of Surolia *et al.* (1973). Concanavalin A-sepharose 4B column wi prepared by cyanogen bromide activation of Sepharose 4B by the procedure c Bishayee and Bachhawat (1974). The concanavalin A content of the column wa 6-8 mg/ml of Sepharose 4B.

Polyacrylamide gel electrophoresis

The purity of the enzyme protein was evaluated by electrophoresis on 7.5% (w/v polyacrylamide gels as described by Davis (1964) at pH 4.3 in β-alanine-acetic aci buffer and at pH 8.3 in Tris-HCl buffer. Each tube contained 25-50 μg of protein The protein was stained with Coomassie brilliant blue R and destained witt methanol: acetic acid: distilled water (1:1.5:17.5, v/v) (Weber and Osborn, 1969) Protein was estimated with crystalline serum albumin as standard by the method o: Lowry *et al.* (1951). The molecular weight of the enzyme was estimated by ge filtration on Sephadex G-200 column according to the procedure of Andrews (1964). The column was calibrated with the following standard proteins, cytochrome C (12,500), soybean trypsin inhibitor (21,500), ovalbumin (45,000), bovine serum albumin (67,000), aldolase (158,000) and catalase (240,000). The neutral sugar was estimated with galactose as standard by the phenolsulphuric acid method of Dubois *et al.* (1956).

Enzyme assay

The standard assay system for the acid α-D-mannosidase contained 200 μmol of citrate-phosphate buffer (pH 4.2), 0.5 μmol of *p*-nitrophenyl-α-D-mannose, 100 μg of bovine serum albumin (containing no enzyme activity), 1 μmol zinc sulphate and the enzyme in a final volume of 0.5 ml. After incubation at 37°C for 30 min, the reaction was stopped by heating the tubes at 100°C for 30 sec. The tubes after cooling were mixed with 2.5 ml of 0.4 M glycine/NaOH buffer (pH 10.5) and centrifuged for 5 min at 2000 *g*. The yellow colour was measured at 405 nm in Spectronic 20. One unit of enzyme was defined as the amount required to liberate 1 nmol of *p*-nitrophenol per min. Other glycosidases namely, α-L-fucosidase, β-D-galactosidase, α-D-galactosidase, α-D-glucosidase and β-D-hexosaminidase were assayed according to the method of Bossmann (1972).

Purification of the enzyme

The human placental tissues were collected in ice and freed from chord tissue and membrane. The tissue was thoroughly washed in cold distilled water and cut into small pieces and kept frozen at −20°C until use. All operations were carried out at 0-4°C unless otherwise mentioned. The pH of the solution was always maintained at 7.0 with dilute ammonia solution during ammonium sulphate precipitation. Frozen tissue (500 *g*) were homogenized with 2500 ml of 0.02 M phosphate buffer, pH 7.0 containing 0.1 M NaCl in SORVALL Omnimixture for 3 min at full speed. The homogenate was stirred for 30 min and centrifuged at 20,000 *g* for 20 min in SORVALL RC-5B. The precipitate was discarded. Solid ammonium sulphate (490 *g*/litre) was added to the supernatant with constant stirring. After 30 min of stirring, the suspension was centrifuged as before and the precipitate was dissolved in 235 ml of 0.02 M phosphate buffer, pH 6.0 containing 0.1 M NaCl. The enzyme solution was then dialyzed against 3000 ml of 0.05 M phosphate buffer, pH 7.0 containing 0.1 M NaCl with 2 changes for 16 h. The precipitate formed during dialysis was discarded by centrifugation as before. The NaCl concentration of the enzyme solution was increased to 0.5 M by the addition of solid NaCl.

Concanavalin A-sepharose 4B column (1.4×14 cm) was equilibrated with 0.05 M phosphate buffer, pH 7.0 containing 0.5 M NaCl and the enzyme solution was adsorbed on the column at a flow rate of 20 ml/h. The column was washed with 0.05 M phosphate buffer, pH 7.0 containing 1 M NaCl at 25°C until the effluent had an absorbance of less than 0.05 at 280 nm. The enzyme was eluted at 25°C with 0.05 M phosphate buffer, pH 7.0 containing 1 M NaCl and 0.5 M α-methyl-D-glucoside and 10 ml fractions were collected. Active fractions (3 to 11) were pooled and dialyzed for 16 h against 100 vol of 0.02 M phosphate buffer pH 7.0 with 3 changes. The enzyme was precipitated from the dialyzed solution by the addition of solid ammonium sulphate (490 g/litre) with constant stirring. The enzyme was collected after 30 min stirring by centrifugation as before. The precipitate was dissolved in a minimum volume of 0.02 M phosphate buffer, pH 7.0. It was dialysed against 50 vol of the same buffer with 4 changes for 24 h. A DEAE-Sephadex A-50 column (1.6×12 cm) was equilibrated with 0.02 M phosphate buffer pH 7.0 and 10 ml of the dialysed enzyme solution containing 100-110 mg of protein was subjected to ion-exchange chromatography on this column.The column, after the adsorption of the enzyme was washed with 50 ml of equilibrating buffer at a flow rate of 15 ml/h. The enzyme was eluted with the same buffer containing 0.05 M NaCl and 3 ml fractions were collected. Active fractions (4 to 16) were pooled and the protein was precipitated by the addition of solid ammonium sulphate (560 g/litre) with constant stirring. The enzyme was collected after 30 min stirring by centrifugation as before. The precipitate was dissolved in a minimum volume of 0.02 M phosphate buffer, pH 6.0 containing 0.1 M NaCl and was dialysed against 500 ml of 0.02 M phosphate buffer, pH 7.0 containing 0.1 M NaCl with 4 changes for 10 h.

The enzyme solution containing 8-10 mg protein in 6-8 ml of total volume was passed through a Sephadex G-200 column (2×75 cm) which was equilibrated and

eluted with 0.02 M phosphate buffer, pH 7.0 containing 0.1 M NaCl at a flow rate c
10 ml/h and 3 ml fractions were collected. The active fractions (12-22) wer
pooled and concentrated by ultrafiltration through a PM-10 membrane. Th
enzyme solution was made 1 mM with respect to zinc ion by the addition of 0.01 N
zinc sulphate solution and heated at 65°C for 1 h. The precipitated protein wa
discarded by centrifugation at 20,000 g for 30 min. The supernatant, containin
the enzyme was concentrated by ultrafiltration through a PM-10 membrane. Th
enzyme when kept at −20°C was stable for at least 8 months.

Results and discussion

Purification of human placental tissue acidic α-mannosidase

The acidic α-mannosidase from placental tissue was purified 4400-fold (table 1)

Table 1. Purification of α-mannosidase.

Steps	Total units	Total protein (mg)	Sp. act.	% Recovery
Homogenate	27,520	33,560	0.8	100
Supernatant	24,348	23,411	1.5	89
Ammonium sulphate	21,320	14.026	1.5	78
ConA-Sepharose eluate	18,667	438	42.5	68
DEAE Sephadex	12,806	124	103.3	47
Sephadex G-200	9,540	8.9	1077.0	35
Heat treatment at 65°C	7,747	2.2	3603.0	28

Since the chromatography on concanavalin A-Sepharose was utilised at an earl
stage there was no neutral α-mannosidase activity. The ion-exchange chromatc
graphy was done at pH 7.0 on DEAE-Sephadex A-50. The enzyme did not resolv
into multiple forms as was the case with human liver (Phillips *et al.*, 1975) an
human kidney (Marinkovic and Marinkovic, 1976) α-mannosidases. The onl
difference was that in earlier cases the ion-exchange chromatography was done a
pH 6.0. In the present case, we found that the enzyme was not retained at pH 6.C
but that it was retained at pH 7.0. The enzyme was free from other acidi
glycosidase activity after Sephadex G-200 gel filtration except that for β
hexosaminidase activity. Hexosaminidase activity was completely removed b
heat treatment at 65°C for 1 h at pH 6.0 in the presence of zinc ions.

pH optimum and kinetic constants

The enzyme from the human placental tissue had optimum activity at pH 4.2 unde
standard assay conditions.

The K_m value obtained with *p*-nitrophenyl α-D-mannoside as substrate by th
plot S/v against S was 1.9 mM and V_{max} was 105.5 nmol/min/mg protein. Thi
K_m value was of the same order as obtained with human kidney and human live
enzyme (Phillip *et al.*, 1976; Marinkovic and Marinkovic, 1976).

Molecular weight

The molecular weight of placental α-mannosidase determined by Sephadex G-200 gel filtration was found to be 300,000 while that of the kidney enzyme was 180,000; that of the liver A form was 220,000 and of the B-form was 300,000 (figure 1).
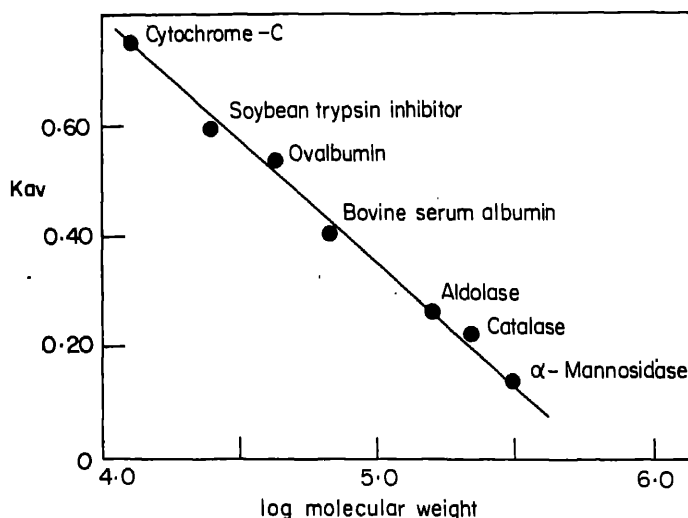

Figure 1. Determination of molecular weight of α-mannosidase by gel filtration on Sephadex G-200.

Effect of zinc and other metal ions

Snaith and Levvy (1968) showed that zinc ions were required for the maximal activity of α-mannosidase from rat epididymis. Snaith (1975) characterised the same enzyme from Jack bean meal as a metalloprotein containing 2 atoms of zinc/ mol of enzyme of molecular weight 230,000. In the present case the enzymic activity of the placental homogenate was completely dependent on zinc ion as was shown by Phillips *et al.* (1976) with human liver enzyme. However, the purified enzyme was only marginally activated (20 to 30%) by zinc ions in the concentration range 20 to 600 mM. At present we are unable to offer any explanation for this behaviour of the enzyme.

In a separate set of experiments the purified enzyme was preincubated for 30 min at 25°C in pH 4.0 buffer in the presence of EDTA (4-250 mM), the enzymic activity was found to be 72 to 85% of the original. Several other metals like Hg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} had no effect at 1 mM concentration, but Ag^{1+} ion at a similar concentration inhibited upto 60% of the activity in the absence of EDTA. The enzyme was protected by Zn^{2+} against inactivation on heating at 65°C for 1 h. Sodium chloride and sodium acetate (1 to 5 mM) had no effect on the enzyme activity.

Electrophoresis

The enzyme on electrophoresis at pH 4.3 on 7.5% and 5% acrylamide gel showed a single band when stained for protein.   But the same protein on electrophoresis between pH 7.00 and 8.3 on 5%, 7.5% and 10% acrylamide did not move from the top of the gel, except for a slight diffusion.

Carbohydrate content

It is well established that lysosomal acid glycohydrolases are glycoprotein.   The glycoprotein nature of α-mannosidase of placental tissue was indicated by its binding to concanavalin A-Sepharose column and subsequent elution with α-methylglucoside.   The neutral sugar content of the purified protein was 10.6%.

Acknowledgements

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Changes in DNA, RNA, protein and the activities of acid and alkaline DNases in developing and aging rat cerebellum

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Abstract. The content of DNA, RNA and protein in cerebellum at different stages of the life span of rat as well as the ratios of protein to DNA, showed that in this region extensive cell proliferation occurs between the 1st and 7th day after birth and once again between the ages of 225 and 750 days. The putative DNA degrading enzymes, acid and alkaline DNases, showed a positive correlation with the rapid DNA accretion noticed during developmental stages as well as during old age. From these results, it could be presumed that there was a second bout of glial cell multiplication in aging cerebellum and that DNases must be playing some important role in the process.

Keywords. DNA; DNases; aging; rat; cerebellum.

Introduction

This laboratory, for the past several years, has been engaged in studying biochemical aspects of developing and aging brain in different species (Subba Rao and Janardana Sarma, 1972; Subba Rao, 1973; Shrivastaw and Subba Rao, 1975; Subba Rao and Shrivastaw, 1976; Subba Rao and Janardana Sarma, 1976; Subba Rao and Shrivastaw, 1979). During our studies on chick brain it was observed that the DNA content of the brain continues to increase even beyond the adult stages indicating that there was cell proliferation occurring even in that adult brain. It also became clear during these studies that the putative DNA degrading enzymes, the acid and alkaline DNases, exhibited maximum activity during the embryonic stages of the brain. Thus these enzymes, although degradative in nature, showed a positive correlation with the DNA synthesis.

This study was undertaken to check whether a similar pattern of biochemical changes would be observed in another species, the rat. In view of the complexity of the type of cells that are present in the brain (Dunn and Bandy, 1974) it was considered appropriate that different regions of the rat brain be examined separately.

Materials and methods

Rats of specified age (Wistar strain) were obtained from Indian Drugs and Pharmaceuticals Limited Animal House, Hyderabad. Highly polymerized calf

thymus DNA, yeast RNA and bovine serum albumin were purchased from S
Chemical Company, St. Louis, Missouri, USA.   All the reagents used were o
Analytical grade.

Cerebellum was separated from the brain and 10% (w/v) homogenates
prepared in glass distilled water using Potter-Elvehjem homogenizer. A po
was immediately taken for the estimation of DNA and RNA (Schmidt
Thannhauser, 1945, Munro 1966).   DNA was estimated by the diphenylaı
method while RNA was measured by the orcinol reaction.   Acid and alkɛ
DNases were assayed as described by McDonald (1955), by measuring the
soluble deoxyribose at the end of incubation.   The activity was also checke
following the increase in ultra violet absorption at 260 nm of the acid sol
fraction. Protein was estimated by the biuret method (Gornall *et al.,* 19
Phosphorus was measured by the procedure of Bartlett (1959).

Results and discussion

There was a steady growth in the wet weight of the cerebellum with advanı
age upto 60 days. This weight was maintained constant upto 225 days but betw
225 and 750 days there was again a significant increase in the weight (table 1)

Table 1. Changes in wet weight, DNA, RNA and protein levels in aging and developing raı
cerebellum

Age (days)	Wet wt. (mg)	DNA/g (mg)	Total DNA (mg)	RNA/g (mg)	Total RNA (mg)	Protein/g (mg)	Total pro (mg)
1(6)	24±1	5.75±0.2	0.13±0.01	5.73±0.50	0.13±0.01	69.8± 3.6	1.62±0.
7(12)	55±4	9.53±0.9	0.53±0.07	6.62±0.49	0.37±0.03	75.3± 4.3	4.18±0.
15(6)	210±40	5.32±1.2	1.20±0.30	4.03±1.10	0.80±0.20	104.6±13.5	22.50±3.
60(10)	240±40	4.40±1.1	1.10±0.28	1.98±0.22	0.49±0.05	126.4±25.4	32.00±6.
225(16)	240±50	2.70±0.5	0.82±0.28	2.40±0.25	0.59±0.09	107.4±17.3	25.20±4.
750(17)	290±30*	5.41±0.9*	1.60±0.16*	2.76±0.26	0.79±0.07*	114.5±15.4	32.62±3.

All the values are expressed as mean ±S.D. and the number of samples analyzed is indicɛ
in paranthesis. For other details see text.

* These values are significantly different from the corresponding values at 225 days of
(P<0.001)

It can be seen from the table that total DNA content of cerebellum increasec
fold between day 1 and 7 after birth, while during the same period the increase
protein content was only 2.5 fold and that of RNA was about 3 fold.   This patte
suggests higher rate of cell proliferation as compared to the increase in cell s
during this period.   Similar higher rate of cell proliferation (as indicated by Dⵏ
increase) in the cerebellum during the few days after birth in rat was observed

earlier workers (Balazs and Patel, 1973; Weichsel, 1974; Griffin *et al.,* 1977; Clark and Weichsel, 1977; Gaitonde *et al.,* 1978; Clark *et al.,* 1978; Litteria, 1980). However, from the 7th day onwards the rate of increase in protein was significantly higher than that of DNA indicating that beyond the 7th day the extent of cell size increase was greater than that of cell proliferation. From the 15th day the levels of DNA remained essentially constant upto 225 days but between 225 and 750 days there was once again a significant increase in DNA content (expressed either as total content or per *g* of the region). In fact the values found in old cerebellum were the highest compared to the values at any other earlier ages. Even in the case of RNA and protein, there was a significant increase in their total content between 225 and 750 days although the values in old cerebellum were not the highest as compared to the values at earlier ages. It should also be noted that the magnitude of accumulation of DNA (100%) between adult and old ages was much greater than those in the case of either RNA or protein (30% and 20% respectively) during the period. These observations point to a second peak of cell proliferating activity during the life span of rat cerebellum confirming our earlier similar findings in the case of chick cerebellum (Subba Rao and Shrivastaw, 1976). The above observation is also in agreement with the other findings with mouse cerebellum (Caron and Unsworth, 1978).

Highest specific activity of acid DNase was found on the 1st day postnatal. The values decreased steadily with increasing age upto 225 days. But there was a significant increase in this value by the time the animal became 750 days old, although this value was much lower than the activities observed during early developmental stages (days 1 and 7). A similar increase in the total activity of acid DNase between 225 and 750 days could also be seen and the value at 750 days was the highest (table 2). Previous studies from this laboratory with chick cerebellum showed that there was no increase of either the specific activity or of total activity of acid DNase in old age (Subba Rao and Shrivastaw, 1976). It thus appears that there is some species variation as far as the activity of acid DNase in aging brain is concerned.

On the other hand, highest specific activity and total activity of alkaline DNase were observed in aging cerebellum. The specific activity remained at the same level from day 1 to 225 days with an approximately 100% increase between 225 and 750 days. A similar magnitude of increase is also found in total activity during this period. The values between 15 days and 225 days showed some fluctuations but it should be noted that the value at 750 days was significantly higher even when compared to the earlier highest activity found at 60 days. It, therefore appears that alkaline DNase may have an important role, in aging cerebellum. These observations are once again in line with our results with chick brain (Shrivastaw and Subba Rao, 1975; Subba Rao and Shrivastaw, 1976).

The ratios of protein, RNA and the activities of DNases to DNA are presented in table 3, since such an expression would indicate indirectly the increase in cell size and the enzyme activities per cell. There was actually a decrease in protein/DNA and RNA/DNA values between days 1 and 7 thereby indicating once again (table 1)

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Table 2. Acid and alkaline DNase activities in aging and developing rat cerebellum.

Age (days)	Acid DNase		Alkaline DNase	
	Sp. activity	Total activity	Sp. activity	Total activity
1(6)	17.0±1.4	27.7±3.4	8.7±0.3	14.0±1.0
7(12)	15.7±1.5	65.7±5.3	9.3±1.0	38.9±5.9
15(6)	4.9±0.6	110.0±27.0	7.6±2.3	204.2±50.9
60(10)	3.0±0.7	92.5±12.4	8.1±1.3	264.8±47.2
225(16)	3.2±0.6	81.3±1.6	7.8±1.6	200.4±36.6
750(17)	5.5±0.7*	176.5±23.0*	14.6±3.1*	445.0±107.0*

For the assay of acid DNase the reaction mixture consisted of 2 mg of highly polymerized calf thymus DNA, in 1 ml of water, 1.5 to 1.7 ml. of 0.1M acetate buffer, pH 5.1, and 0.3 to 0.5 ml of the cerebellar homogenate. At the end of a 2 hour incubation at $37^{\circ}C$ the reaction was terminated by adding 2 ml of 1.4M perchloric acid and immediate chilling. The whole mixture was filtered through Whatman 42 filter paper and deoxyribose content/increase in UV absorption at 260 nm was estimated in the filtrate. For measuring alkaline DNase activity the procedure is same as for acid DNase except that the reaction was carried out at pH 8.2 (0.05M Tris-Hcl buffer) and denatured DNA (2 mg) instead of Native DNA was used as substrate. Specific activity is expressed as µg of DNA-P liberated per 2 h per mg of protein. Total activity is obtained by multiplying the specific activity with the total amount of protein in mg. All the values are expressed as means ±S.D. and the number of samples are indicated in the bracket.

* These values are significantly different from the corresponding values at 225 days of age ($P<0.001$)).

Table 3. Protein/DNA, RNA/DNA and activities of DNases/mg of DNA in developing and aging rat cerebellum.

Age (days)	Protein/ DNA	RNA/DNA	Acid DNases/ mg DNA	Alkaline DNase/ mg DNA
((1(6)	11.9±0.6	1.0±0.1	203.50±22.2	102.2±3.9
7(12)	8.0±0.06*	0.7±0.06*	131.8±14.1*	73.6±7.3*
15(6)	20.7±4.8	0.8±0.2	98.2±16.0	139.0±40.2
60(10)	30.7±4.1	0.7±0.2	94.8±13.4	258.6±64.6
225(16)	28.8±5.0	0.7±0.2	106.0±22.0	213.9±65.0
750(17)	21.1±2.4**	0.5±0.1	112.6±14.5	316.6±41.7**

All the values expressed as means ±S.D. and the number of samples are indicated in the bracket.

* These values are significantly different from the corresponding values at 1 day of age ($P<0.001$)

** These values are significantly different from the corresponding values at 225 days of age ($P<0.001$)

that the rate of DNA accumulation (hence cell proliferation) was higher than the rate of either protein or RNA accumulation (cell size increase) during this period. From the 7th day onwards both protein/DNA and RNA/DNA increased and these adult levels were maintained upto 225 days. Between 225 and 750 days there was a small but statistically significant decrease in protein/DNA value whereas the RNA/DNA ratio showed no change (although there was an apparent difference between the values, they were not statistically significant). The absence of a difference in RNA to DNA ratio between adult and aging cerebellum was in agreement with the earlier findings with mouse cerebellum (Chaconas and Finch, 1973; Somarjski and Rolsten, 1973). However, the studies on RNA/DNA ratios in different regions (except Corpus striatum) of rat brain by Shaskan (1977) showed an increase in this ratio between the adult and senescent rat brains. But, in this study the cerebellum was not analyzed while the present investigation deals exclusively with the changes in cerebellar region and it is therefore difficult to compare the two data. The decreased protein/DNA value with a simultaneous increase in total DNA content observed in the present studies (table 1) in aging cerebellum strongly suggest a rapid cell proliferating activity during this period.

Acid DNase activity per mg of DNA was highest at the earliest age period studied with a decrease of this value to a lower adult level subsequently. No change in this adult level activity could be noticed during old age (table 3). However, alkaline DNase activity expressed per mg of DNA was highest in old age (750 days). There was a significant increase in this activity between 225 and 750 days, thereby indicating that this enzyme protein is probably synthesized in larger amounts in aging cerebellum. On the basis of these results, it is tempting to speculate that acid and alkaline DNases, in particular the latter, may be playing a vital role in aging rat cerebellum. Further work could throw some light on this intriguing aspect.

Acknowledgement

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Extractability of polyphenols of sunflower seed in various solvents

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Abstract. The extractability of chlorogenic acid from defatted sunflower seed flour in water and salt solutions at different pH values and also in aqueous organic solvents was determined. It increased with increase in pH and at pH 8 in water nearly 70% chlorogenic acid was removed in a single extraction, while NaCl did not increase the extraction, and, MgCl₂ and CaCl₂ increased it, especially at higher concentrations. Methanol, ethanol, isopropanol and acetone, at 20% concentration in water, caused the maximum extraction of polyphenol. These organic solvents without added water were poor solvents for the extraction of polyphenol from the flour.

Keywords. Polyphenol; sunflower seed; extractability of chlorogenic acid.

Introduction

The presence of chlorogenic acid as a major polyphenol in sunflower seeds has been well recognised (Smith and Johnsen, 1948; Joubert, 1955; Sechet *et al.*, 1959; Milic *et al.*, 1968). Caffeic acid and quinic acid are also present in sunflower seeds (Sechet *et al.*, 1959; Milic *et al.*, 1968). These cause discolouration of sunflower proteins at alkaline pH (Smith and Johnsen, 1948) and also lower the nutritive value (Horigome and Kandatsu 1968; Jung and Fahey, 1981) because of their interaction with residues such as lysine, cysteine, methionine (Pierpoint, 1970), alanine, phenylalanine and glutamic acid (Nakatani and Kurasawa, 1979). Many methods have been tried to remove the polyphenols from sunflower seed using aqueous, organic and aqueous—organic solvents (Smith and Johnsen, 1948; Joubert, 1955; Mikolajczak *et al.*, 1970; Pomenta and Burns, 1971; Sosulski *et al.*, 1972, 1973; Fan *et al.*, 1976; Sodini and Canella, 1977). Reducing agents have also been used to prevent oxidation of chlorogenic acid during isolation of the proteins (Gheyasuddin *et al.*, 1970). In these methods complete removal of chlorogenic acid is not achieved in a single extraction and repeated extraction is necessary to achieve even partial removal. The hydrogen bond between hydroxyl groups of phenolic compounds and peptide bonds in protein is known to be unusually strong and equilibrium in aqueous solutions strongly favours the formation of complexes between phenols and proteins (Loomis and Battaile, 1966).

Inspite of a large number of methods for removal of polyphenols (especially chlorogenic acid) from sunflower, no systematic attempt appears to have been made to study the extractability of the polyphenols in various aqueous and

aqeuous-organic solvents as a function of solvent composition, pH, temperature, etc. The present investigation attempts such a systematic study. The choice of the solvent system has been guided by the solubility of the polyphenols in various solvents as indicated in the literature.

Materials and methods

Materials

Sunflower: Sunflower seeds, the Russian variety EC 68415, grown in the State of Karnataka, were obtained from Karnataka State Agro Seed Corporation, Mysore.

Chemicals: Chlorogenic acid, caffeic acid and quinic acid used as standards were from Sigma Chemical Co., St. Louis, Missouri, USA; NaCl, $CaCl_2$, $MgCl_2$, $NaNO_2$ and CH_3COOH were of GR grade from E. Merck, Dermstadt, Germany. Isopropanol, acetone, methanol and ethanol were from British Drug House Ltd., and were distilled once before use.

Methods

Sunflower seeds were dehulled in a centrifugal disc huller (Sastry, 1978) after drying at 50°C for 2 h. The kernels were flaked and defatted with *n*-hexane and the residual solvent was removed by air drying for 4-6 h at 30°C.

Estimation of protein

The nitrogen content was determined by microKjeldahl method (AOAC, 1975) and converted to crude protein by using a factor of 6.25.

Estimation of chlorogenic acid, caffeic acid and quinic acid: Defatted flake was ground in a Waring Blendor to pass 40 mesh sieve. Two g of the sample was used for the estimation of the acids according to the procedure of Pomenta and Burns (1971).

Since it was observed that chlorogenic acid was the major polyphenol (about 66%) in sunflower seed, to determine the efficiency of polyphenol removal from sunflower meal by various treatments only chlorogenic acid was estimated.

Removal of polyphenols

The following general procedure was used. The defatted sunflower meal (5 g) of 10 mesh sieve size were mixed with solvent in the ratio of 1 of flour to 10 of solvent (w/v) and stirred for 2 h at room temperature (about 28°C). The slurry was filtered and the residue on the filter paper was dried at 55-60°C in an oven till constant weight was obtained (5-6 h). The chlorogenic acid content of the residue was determined by the method of Pomenta and Burns (1971).

The effect of extraction with aqueous methanol, ethanol, isopropanol and acetone whose composition varied from 0-80%; NaCl, $CaCl_2$ and $MgCl_2$ solutions of varying molarity, water at different pH's and temperatures on removal of polyphenol was determined. The pH of water was adjusted with 1M HCl or 1M

NaOH. For determining the effect of temperature on the extractability of chlorogenic acid by water a shaking water bath whose temperature was controlled to $\pm 1^\circ$C of the desired temperature was used.

When extractions were done in salt solutions, the residue after drying would contain the corresponding salt. Since the salts used were chlorides of different cations, the chloride content of the residue was estimated (Vogel, 1961), and the weight of the residue was corrected for the salt content.

Results and discussion

The chlorogenic, caffeic and quinic acid contents of sunflower meal given in table 1

Table 1. Composition of phenolic acids of defatted sunflower flour*.

Composition	g/100 g of flour
Protein	54.0
Chlorogenic acid	1.86
Caffeic acid	0.56
Quinic acid	0.39

* moisture free basis

indicate that chlorogenic acid forms the major polyphenol in the meal. The chlorogenic acid content of the meal was in the range of values of 1.42 to 4.00% reported for a number of sunflower varieties (Dorrell, 1976).

Only about 30% of chlorogenic acid was extracted in the pH range 2-3 (figure 1).
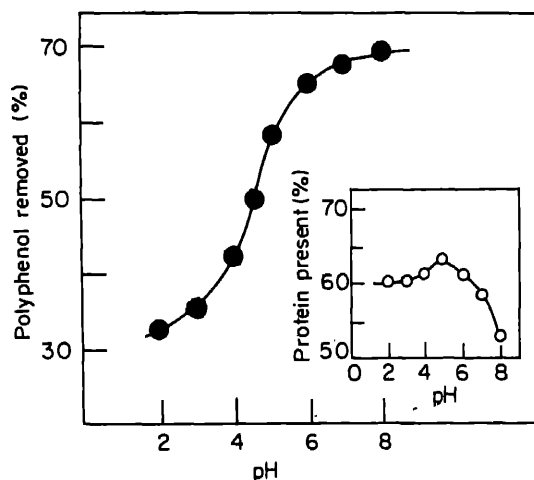
Figure 1. Effect of pH on the extractability of polyphenol in water. Inset figure gives the protein content of the flour.

However, above pH 4 there was a steep increase in the extractability of chlorogenic acid and reached a value of about 70% around pH 8. Polyphenols are known to undergo oxidation to the corresponding quinones at alkaline pH (Sondheimer, 1964). It is possible that the higher percentage of polyphenols extracted at alkaline pH could be due to the oxidation of the polyphenol and their inability to be estimated by the method of Pomenta and Burns (1971). To clarify this point the following experiments were done. The pH of a solution of chlorogenic acid in water was adjusted to different values upto pH 10 and set aside for 30 min at room temperature and the chlorogenic acid content was estimated (Pomenta and Burns, 1971). It was observed that upto pH 8 there was no change in the absorbance of solutions. However, above pH 8 the significant change in absorbance indicated that chlorogenic acid could not be estimated by the method of Pomenta and Burns (1971). This experiment clearly demonstrated that the data on the extractability of chlorogenic acid upto pH 8 were reliable and the data in figure 1 is not vitiated by any artifacts of assay. Thus it can be concluded that at pH 7 nearly 70% of chlorogenic acid could be removed by a single extraction.

The protein content of the meal which had been subjected to polyphenol extraction was also estimated. Upto pH 7 there was not much loss in the protein content of the meal (figure 1, inset). However, above this pH value there was considerable extraction of the protein also. These results indicate that by extraction at pH 6 to 7 most of the chlorogenic acid can be removed without loss in protein content.

The effect of NaCl, CaCl$_2$ and MgCl$_2$ was also tried on the a extractability of chlorogenic acid. The extraction with 0.1 M, 0.5 M and 1 M NaCl at different pH values did not result in increased removal of chlorogenic acid, but the amount extracted was lower. On the other hand, the addition of salt lead to greater losses in the protein content (figure 2, inset). This clearly suggested that extraction with
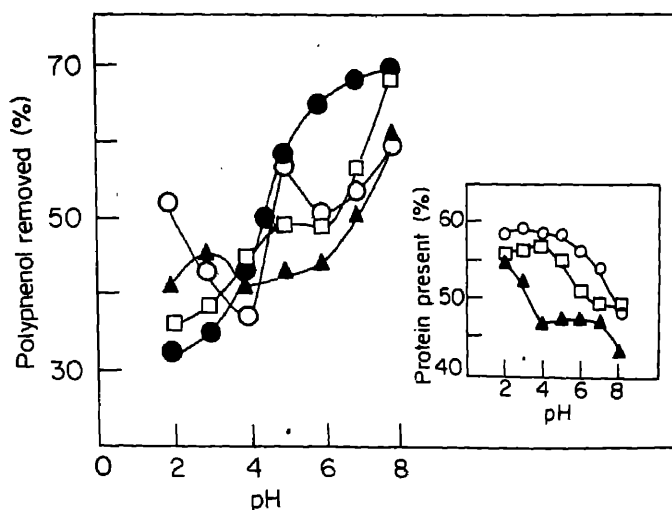
Figure 2. Effect of pH on the extractability of polyphenol at various concentrations of NaCl: (O) 0.1 M NaCl; (□) 0.5 M NaCl; and (▲) 1 M NaCl. Inset figure gives the protein content of the flour. (●) indicates the effect of pH on the extractability of polyphenol in water alone.

salt solutions at different pH offers no advantages over the use of water. The result obtained with 0.05 M and 0.4 M MgCl$_2$ salts is given in figure 3. While at
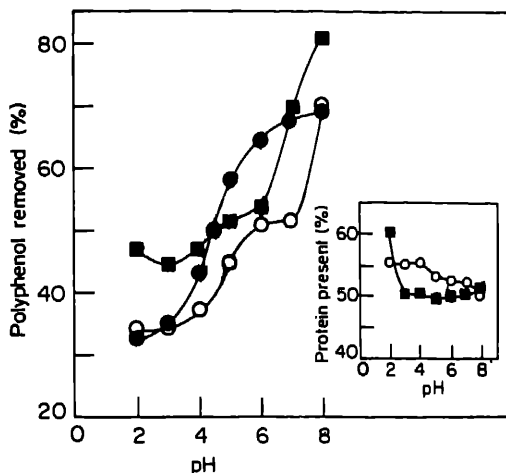
Figure 3. Effect of pH on the extractability of polyphenol at various concentrations of MgCl$_2$: (O) 0.05 M MgCl$_2$ and (■) 0.4 M MgCl$_2$. Inset figure gives the protein content of the flour. (●) Indicates the effect of pH on the extractability of polyphenol in water alone.

low MgCl$_2$ concentration there was no marked increase in chlorogenic acid extraction at a higher MgCl$_2$ concentration the extractability of polyphenols increased. In fact, at pH 8 nearly 82% of the polyphenol could be removed at 0.4 M MgCl$_2$. However, the extractability of the protein also increased and the protein content of the meal was much lower than that of the untreated meal. Thus while there was an advantage in the removal of the polyphenol there was a disadvantage so far as the protein content of the meal was concerned. Similar results were obtained with CaCl$_2$ also (figure 4). Here, in fact the extractability of the protein
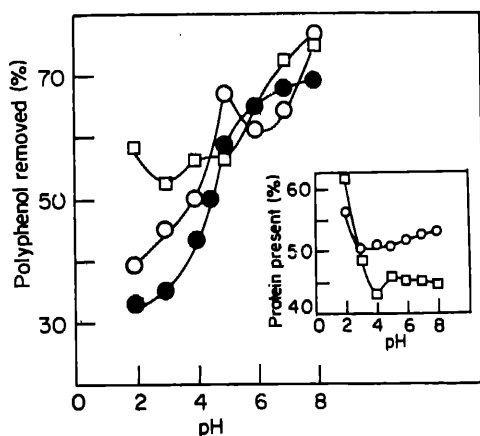
Figure 4. Effect of pH on the extractability of polyphenol at various concentrations of CaCl$_2$: (O) 0.1 M CaCl$_2$ and (□) 0.5 M CaCl$_2$. Inset figure gives the protein content of the flour (●) Indicates the effect of pH on the extractability of polyphenol in water alone.

from the meal was much higher and there was considerable loss in the prc
content.

The greater efficiency of salts, especially $MgCl_2$ and $CaCl_2$ to remove chloroge
acid from sunflower flour may be due to their ability to interfere with any ic
linkages that may be formed between this acid and the protein. This inferenc
supported by the observation that increase in the salt concentration increases
efficiency of extraction and also the divalent salts have a greater efficiency than
monovalent salts like NaCl.

In figure 5 the effect of temperature on the extraction of chlorogenic acic
given. In the temperature range 30-50°C there was an increase in the extractabil
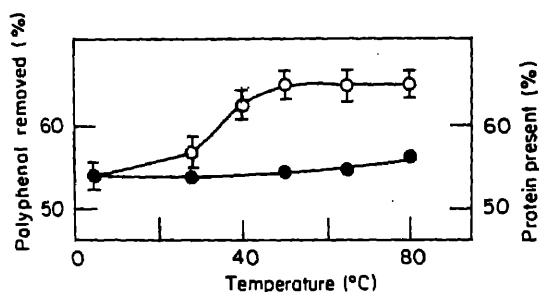
Figure 5. Effect of temperature (°C) on the extractability of polyphenol (Ō). Protein
content of the flour after treatment with each temperature is also shown (●).

of chlorogenic acid; it increased from about 55% to about 65%. However, betwee
50-80°C there was no improvement in the extractability. The protein content c
the meal also was more or less constant upto 65°C. At 80°C the protein conter
was slightly higher (56%). The increased extractability of chlorogenic acid as th
temperatures increased is in accordance with the observation of Sosulski *et a*
(1972) who carried out the extraction only at 60°C and 80°C. However, thi
contradicts our observation that between 50-80°C there was no increase ii
extractability. This difference could possibly be due to differences in the varietie:
of sunflower, and also due to the difference in the technique used for extracting the
polyphenols.

In figure 6 the extractability of chlorogenic acid from sunflower flour in aqueous·
organic solvents is given as a function of the percentage of the organic solvent in
the aqueous-organic solution. In methanol, ethanol, isopropanol and acetone the
extractability increased upto about 20% and remained constant upto about 80%.
However, above this concentration the extractability of·chlorogenic acid decreases
and in absolute ethanol and isopropanol the extractability was very poor being of
the order of 10-12%. In·pure methanol and acetone the extractability was slightly
better (30%). These results clearly suggested that use of higher concentrations of
alcohol did not offer any advantage in extracting chlorogenic acid. Joubert (1955)
used 50% ethanol to extract the polyphenols. Smith and Johnsen (1948) used 70%
ethanol or near absolute methanol to remove the acid. Similarly Fan *et al.*, (1976)
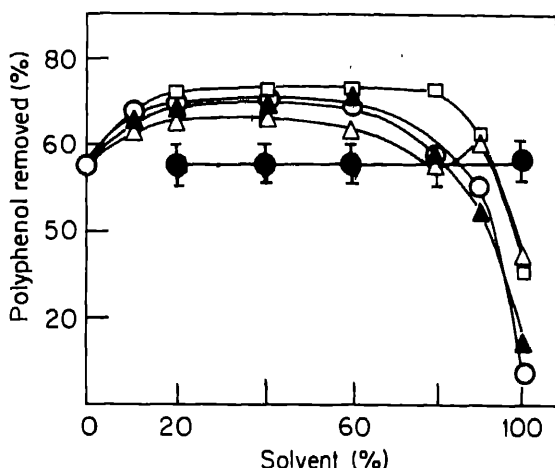
Figure 6. Effect of various organic solvents and various percentages aqueous-organic solvent mixtures in the extractability of polyphenols; (O) ethanol; (□) acetone; (▲) isopropanol; (Δ) methanol and (◑) residual proptein in the flour after treatment with the above solvents respectively.

used 70% ethanol at 24°C. These authors used repeated extractions to achieve complete removal of polyphenol. These authors do not state why this composition of the solvents was used for extracting the polyphenols. Our results suggest that use of lower concentration of alcohol has an advantage over the use of higher concentrations. The use of lower concentrations of alcohol has the added advantage that the proteins are not likely to be denatured and possibly their physico-chemical and functional properties are not impaired. Figure 6 also shows that the protein content of the flour was not decreased by extraction at low concentrations of alcohol.

The low solubility of the polyphenols in absolute organic solvents may be due to strengthening of the hydrogen bonds between polyphenols and protein in these solvents (Sabir *et al.*, 1974). On the other hand the increase in solubility upon the addition of water to organic solvents could be due to the weakening of the hydrogen bonds in aqueous solutions. It could also be due to the increase in basicity (Bates *et al.*, 1966; Paabo *et al.*, 1966; Brandes and Stern, 1968; 1976) and increased ionisation of the polyphenols in such solutions.

As mentioned earlier a number of methods have been attempted for removal of the polyphenol from sunflower meals. Sosulski *et al.*, (1972) utilised 0.001N HCl for extraction. The pH of this solution would be approximately 3-4. It is not clear why acid condition was employed for extraction of chlorogenic acid. Our results indicate that extractability of the acid is much better at higher pH values and neutral pH. This is also confirmed by the observation that the binding of chlorogenic acid by sunflower 11S protein is. higher at lower pH than at higher pH (Sastry, Personal communication).

This investigation indicates that water or salt solutions at neutral pH organic solvents in water are efficient solvents for the removal of chlorogei (and possibly caffeic acid and quinic acid) from sunflower meal.

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Immobilization of β-D-galactosidase from *Lactobacillus bulgaricus* on polyacrylamide in the presence of protective agents and properties of the immobilized enzyme

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Abstract. β-D-Galactosidase (EC 3.2.1.23) from *Lactobacillus bulgaricus* (1373) was immobilized in a polyacrylamide gel lattice in the presence of dithiothreitol, glutathione, cysteine, bovine serum albumin, casein, lactose and glucono-δ-lactone. Cysteine, bovine serum albumin, and lactose were found very effective in preserving the activity. With cysteine, bovine serum albumin and lactose, the activity yields were 61, 60 and 66% respectively, as compared to 31% without protective agents. The yield improved upto 85% when all the three protective agents, cysteine, bovine serum albumin and lactose were added during immobilization. The addition of protective agents did not have any effect on optimum pH, optimum temperature, kinetic constants and pH stability when compared with β-galactosidase immobilized without the use of protective agents; however the heat and storage stabilities were found to increase.

Keywords. Immobilized β-galactosidase; *Lactobacillus bulgaricus;* polyacrylamide gel entrapment; protective agents; kinetic constants.

Introduction

In our previous study (Makkar *et al.*, 1981) β-D-galactosidase from *Lactobacillus bulgaricus* was immobilized in a polyacrylamide gel lattice, and the properties of the immobilized and native enzymes were compared. However, the maximum activity yield of immobilized β-galactosidase preparation was very low (31%). A low activity yield during entrapment in polyacrylamide was reported for a number of enzymes (Miyamoto *et al.*, 1977; Ohmiya *et al.*, 1975; Kobayashi *et al.*, 1975; Chibata, 1978). The loss in activity may be due to: a) denaturation by acrylamide in a manner similar to urea and b) oxidation by potassium persulphate (Digani and Miro, 1970; Miyamoto *et al.*, 1977). In the present study efforts have been made to decrease the activity losses by using protective agents (substrate, inhibitor and thiols) during immobilization.

Materials and methods

The materials are the same as those employed earlier (Makkar *et al.*, 1981).

The immobilized β-galactosidase was prepared essentially as described e. (Makkar *et al.,* 1981) with incorporation of different protective agents i: enzyme solution before the addition of enzyme to monomers. The immobiliz galactosidase preparation obtained by the addition of bovine serum albi lactose and cysteine (2.5, 5 and 10 mg respectively per 8 ml) as protective a was used for studying the properties.

The native and immobilized β-galactosidases were assayed as desc previously (Makkar *et al.,* 1981).

Results

Effect of protective agents on activity yield

β-Galactosidase of *L. bulgaricus* requires sulfhydryl groups for activity (unpubl observation). In order to protect—SH groups from oxidation by potassium sulphate, dithiothreitol, glutathione and cysteine were added (table 1). Cys

Table 1. Effect of protective agents during polymerization on the activity of immobilizec galactosidase.

Protective agents	Activity yield* (%) Concentrations of protective agents (mg)		
	2.5	5	10
Dithiothreitol	38	53	55
Glutathione	37	49	50
Cysteine	40	55	61
Bovine serum albumin	60	60	—
Casein	37	37	—
Lactose	66	66	—
Glucono-δ-lactone	—	33	33

Without protective agent the activity yield was 31%
With nitrophenylgalactopyranoside substrate.
—Not determined.

was found to be the most effective protecting agent, enhancing the yield from to 61%. Among the additives tried; bovine serum albumin and lactose were effective, in preventing loss of activity but casein and glucone-δ-lactone v ineffective (table 1).

The activity yields of combinations (bovine serum albumin+lactose) (cysteine+lactose) were comparable but greater than that obtained by indivi protective agents (tables 1 and 2). A mixture of bovine serum albumin

Table 2. Effect of different combinations of bovine serum albumin, lactose and cysteine on the activity of immobilized β-galactosidase.

Protective agents	Activity yield* (%)
Bovine serum albumin + lactose	74
Bovine serum albumin + cysteine	65
Lactose + cysteine	73
Bovine serum albumin + lactose + cysteine	85

The concentration of bovine serum albumin, lactose and cysteine used was 2.5, 5 and 10 mg respectively.

* Using nitrophenylgalactopyranoside as substrate.

cysteine gave a 65% activity yield while the addition of all the three, bovine serum albumin, lactose and cysteine yielded the maximum activity (85%). The properties of the immobilized β-galactosidase obtained by the addition of these three protective agents were studied.

Properties of the enzyme immobilized in the presence of protective agents

Immobilized β-galactosidase had pH and temperature optima at 6.5 and 42°C with both lactose and nitrophenyl-galactopyranoside as substrate in 50 mM phosphate buffer. The K_m values calculated from Lineweaver−Burk plots drawn with lactose and nitrophenyl-galactopyranoside were 9.4 mM and 0.90 mM respectively. Immobilized β-galactosidase started losing lactivity above 45°C when heated for 10 min. The preparation was more stable above 60°C than the native enzyme or the β-galactosidase immobilized without using protective agents. The pH stability of the immobilized β-galactosidase preparation with a protective agent was studied as reported earlier (Makkar *et al.*, 1981). The immobilized β-galactosidase prepared without protective agents showed maximum stability at pH 6.5, and the residual activity obtained at all the pH values was almost the same as that obtained for immobilized β-galactosidase prepared without the use of protective agents (Makkar *et al.*, 1981). Immobilized β-galactosidase was stored at 4 and 25°C. No loss in activity was found even after 30 days of storage at 4°C and after 60 days of storage 80% of the initial activity was found. At 25°C no decrease in activity was recorded upto 20 days of storage. No loss in activity was found in the immobilized preparation after using it 15 times. At 2.5 and 5 h of incubation 45 and 60% respectively of lactose were hydrolysed (Makkar *et al.*, 1981).

Discussion

As anticipated, dithiothreitol, glutathione and cysteine markedly increased the activity yield of immobilized β-galactosidase as sulfhydryl groups are required for the catalytic activity of β-galactosidase from *L. bulgaricus* (unpublished data). The protection of activity in the presence of dithiothreitol, glutathione and

cysteine might be due to the protection of essential sulfhydryl groups of β-ga sidase of *L. bulgaricus* during immobilization. Dithiothreitol has also reported to be effective during immobilization of β-galactosidase from *Aspe oryzae, Kluyveromyces lactis* and *Escherichia coli* K12. However, glutat was effective only for *A. oryzae* and not for *K. lactis* and *E. coli* β-galacto: (Kobayashi *et al.*, 1975; Ohmiya *et al.*, 1975). Bovine serum albumin was fou be very effective in the present study (activity yield 60%). However, in the c β-galactosidase from *A. oryzae*, it is reported to stabilize the immobilized ac only moderately (40%), as against 28% without it (Ohmiya *et al.*, 1975). In th of *E. coli* β-galactosidase, bovine serum albumin increased the activity yielc marginally and for *K. lactis* β-galactosidase, it was not effective (Kobayashi 1975). The presence of substrate (lactose) during immobilization too was effective. This suggests that substrate binding protected the enzyme a inactivation. The presence of substrate during immobilization of hexok (Miyamoto *et al.*, 1977) and aspartase (Tosa *et al.*, 1973) was found to be effe Glucono-δ-lactone, an inhibitor of β-galactosidase, did not increase the ac yield (33%). Similar results have been obtained by Kobayashi *et al.*, (1975) galactosidase of *K. lactis* and *E. coli*. However, for *A. oryzae* β-galactos glucono-δ-lactone was found to be the best amongst all the studied prote agents (Ohmiya *et al.*, 1975). The difference in the degree of protectic different protective agents appears to depend upon the origin of β-galactosi

Table 3. Comparison of properties of native β-galactosidase, immobilized β-galactosida prepared without the use of protective agents and immobilized β-galactosidase prepared wi protective agents.

Properties	Native	Immobilized β-galactosidase (without protective agents)	Immobilized β-galactosidase (with protective agents)
Optimum pH	6.5	6.5	6.5
Optimum temperature	42°C	42°C	42°C
K_m (lactose)	10 mM	9.7 mM	9.4 mM
K_m (nitrophenylgalactopyranoside)	0.94 mM	0.88 mM	0.90 mM
Heat stability (remaining activity %):			
45°C, 10 min	97	97	100
65°C, 10 min	10	19	28
pH stability (maximum stability at pH)	6.5	6.5	6.5
Storage stability (remaining activity %):			
4°C (60 days)	40	70	80
25°C (20 days)	73	100	100
Lactose hydrolysis (%):			
2.5 h	—	20	45
5.0 h	—	30	60

Data for native and immobilized β-galactosidase (without protective agents) taken from earlier paper (Makkar *et al.*, 1981).

The addition of cysteine, bovine serum albumin and lactose gave an excellent activity yield (85%). It is quite likely, in other preparations too the activity yield would increase when different combinations of protective agents were used.

No difference was observed in the optimum pH, optimum temperature, kinetic constants and pH stability between immobilized β-galactosidase prepared with and without protective agents and the native β-galactosidase. However heat stability and storage stability of the preparation made by using protective agents were found to be greater (table 3). The extent of hydrolysis of lactose was greater with the protected immobilized enzyme (table 3). The greater hydrolysis of lactose was obviously due to greater activity yield of the preparation.

Acknowledgements

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Effects of heavy water on mitochondrial respiration and oxidative phosphorylation

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Abstract. Studies on the influence of heavy water on mitochondrial respiration and oxidative phosphorylation revealed that both isotope and solvent effects, may be responsible for the observed changes. Although the two types of effects could not be totally delineated from each other by the experimental approaches employed, the isotope effect appeared to be primarily responsible for the uncoupling of oxidative phosphorylation, while the inhibition of respiration in the presence of ADP (State 3 respiration) could be a manifestation of the solvent effect.

Keywords. Isotope effect; solvent effect; heavy water.

Introduction

Earlier observations indicated the participation of water or the aqueous phase in biological oxidation-reduction reactions. Heavy water and several organic solvents were found to be 'nonspecific' inhibitors of the respiratory chain activity and the inhibitory action could not be attributed to solvent viscosity (Tylor and Estabrook, 1966). Shibata and Watanbe (1949) found that D_2O inhibited several oxidizing enzymes including mushroom cytochrome c oxidase (EC 1.9.3.1).

The respiration of both intact mitochondria and of non-phosphorylating particles [in the presence of ADP+phosphate, or of DNP (Baum and Rieske, 1966; Margolis *et al.,* 1966; Tylor and Estabrook, 1966)] were inhibited by D_2O. It also inhibited the transfer of electrons between cytochromes b and c, in complex III (Baum and Rieske, 1966). The steady-state reduction of nicotinamide nucleotide and the cytochromes in a D_2O-inhibited system and in water (Tylor and Estabrook, 1966) were similar. Heavy water also inhibited the energy linked nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) and the Mg^{2+} stimulated ATPase, (EC 3.6.1.3), but had little effect on the ATP-Pi exchange reaction or on DNP-induced ATPase (Margolis *et al.,* 1966). The P : O ratio of phosphorylating

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Abbreviations used: cyt, cytochrome; ATPase, adenosinetriphosphatase; RCI, respiratory control index.

submitochondrial particles was markedly greater (Margolis *et al.,* 1966) and of the intact mitochondria slightly greater in D_2O than in H_2O. The decrease of the P : 0 ratio brought about by uncouplers was not affected by D_2O (Baum and Rieske, 1966; Laser and Slater, 1960).

The present study was undertaken to examine, in detail, the effects of heavy water on mitochondrial respiration and oxidative phosphorylation to gain some insight into the relationship of the respiratory chain components to their environ- ment. The results suggest that water plays a major role in the interaction of the components of the electron transport chain during oxidative phosphorylation.

Materials and methods

Adenosine triphosphate, cytochrome c, sodium succinate, glucose, trichloroacetic acid, ascorbic acid, tris (hydroxymethyl) aminomethane, ethylenediaminetetra- acetic acid, adenosine diphosphate and hexokinase were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Sucrose and other inorganic chemicals of Analar grade were obtained from British Drug House Ltd., Poole, England.

Male albino rats of Wistar strain, weighing around 120-140 g, and maintained on laboratory stock diet, were used in these studies. Rats were killed by cervical dislocation, the livers were quickly removed, cleaned and chilled in 0.25 M sucrose. Livers were homogenised in 0.25 M sucrose to yield 10% homogenates. Mitochondria were isolated according to the method of Hogeboom (1955) and washed once with 0.25 M sucrose.

In one set of experiments, the mitochondrial pellet was suspended in 0.25 M sucrose in H_2O. The mitochondrial suspension was saturated with oxygen by gently bubbling the gas through it. This has been designated as preparation I.

In another set of experiments the mitochondrial pellet was suspended in 0.25 M sucrose in D_2O and was saturated with oxygen. This suspension was prepared just 10-15 min. before starting the reaction. This has been designated as preparation II.

The method of Yost *et al.,* (1967) was used for manometric determination of P/O ratio. The main vessel of the Warburg flask contained: sucrose, 250 µmol; sodium succinate, 40 µmol; potassium phosphate buffer, pH 7.4 (pD 7.8) 36 µmol; cyt c, 0.09 µmol; ATP, 6 µmol; $MgSO_4$, 15 µmol; potassium fluoride, 65 µmol and hexo kinase 10 mg in 0.15 M glucose. The central well contained 0.1 ml of 5 N potas sium hydroxide along with fluted filter paper strips. The total volume of the reac tion mixture was 3.2 ml. The reaction was carried out at 25°C for 30 min. Oxygen consumption was determined manometrically and esterification of phosphate was determined by measuring orthophosphate before and after the incubation (Lowry and Lopez, 1946).

For the polarographic determination of oxygen uptake and ADP/O ratio, an oxygraph (Gilson Medical Electronics, Wisconsin, USA.) was used. The method followed was that described by Chance and Williams (1955). The reaction medium consisted of 38 mM NaCl; 46 mM KCl; 12 mM KF; 6 mM $MgCl_2$; 16 mM potassium phosphate buffer, pH 7.4 (pD 7.4); 4.8 mM sodium succinate; 0.4 µmol of ADP and 1-1.5 mg mitochondrial protein in a total volume of 2 ml.

The assay of mitochondrial ATPase was carried out by the method of Veldesma-Currie and Slater (1968) with some modifications (Katyare *et al.,* 1971). The reaction system contained 74 mM KCl, 50 mM sucrose, 50 mM Tris-HCl, pH 7.4 0.5 mM EDTA, 8 mM MgCl$_2$, 1.2 to 1.5 mg mitochondrial protein in a total volume of 2.0 ml. The reaction was carried out at 25°C for 15 min and was stopped by the addition of 0.5 ml of chilled 10% trichloroacetic acid (w/v). Appropriate zero time controls were run simultaneously. The tubes were immediately chilled and centrifuged at 3,000 *g* for 10 min.Orthophosphate in the supernatant fraction was estimated by the method of Fiske and Subbarow (1925).

Results and discussion

From table 1, it could be observed that the oxygen consumption with succinate as substrate decreased progressively with increase in the concentration of D$_2$O. With

Table 1. Effect of D$_2$O on mitochondrial (preparation I) respiration and P/O ratio.

D$_2$O (%)	Mitochondrial respiration (μ atoms/h /mg protein)	% Control	Pi esterification (μ moles/h /mg protein)	% Control	P/O
0	1.6±0.5	100	2.9±0.04	100	1.8
20	1.2±0.5	77	2.3±0.01	79	1.9
40	1.2±0.8	75	2.3±0.07	79	2.0
60	1.0±0.4	65	1.9±0.01	65	1.8
80	1.0±0.3	62	1.6±0.01	57	1.7
100	0.8±0.1	49	1.4±0.02	48	1.8

Mitochondrial pellet (approx. 6-8 mg protein) suspended in an incubation mixture (total volume of 3.2 ml) containing the various additions as indicated in text was determined by manometry. The reaction was carried out at 25°C for 30 min. Results are averages of five independent experiments ±SEM.

total replacement of H$_2$O by D$_2$O, oxygen consumption was about 50% of the control value. A similar trend was seen with esterification of Pi and at all concentrations of D$_2$O, the decrease in oxygen consumption was accompanied by a corresponding change in esterification of Pi. As a result, the P/O ratio remained unaltered.

With the mitochondrial preparation preincubated in heavy water 15 min prior to the reaction (preparation II), oxygen consumption even with no D$_2$O (table 2) in the incubation medium, was significantly lower than that of mitochondrial preparation I (table 1). The preincubated mitochondria also showed a gradual decrease in oxygen consumption with increasing concentrations of D$_2$O. A 50% decrease in oxygen uptake over control was observed at $\approx$100% D$_2$O.

The state 3 respiration of mitochondria from preparation I, exhibited an initial enhancement of oxygen uptake with increase in D$_2$O concentration upto 80%, the increase being maximum at a D$_2$O level of 40% (table 3). However, at total

Table 2. Effect of D_2O on mitochondrial (preparation II) respiration and P/O ratio

D_2O (%)	Mitochondrial respiration (μ atoms/h/mg protein)	% Control	Pi esterification (μmol/h/mg protein)	% Control	P
0	1.0±0.02	100	1.8±0.01	100	1
20	0.8±0.08	78	1.4±0.03	79	1
40	0.7±0.02	69	1.3±0.02	71	1
60	0.6±0.07	61	1.1±0.01	62	1
80	0.6±0.04	60	1.1±0.05	60	1
100	0.5±0.01	51	0.9±0.04	50	1.

Experimental details were as shown under Table 1 with the only exception that th mitochondrial suspension was also prepared in 0.25 M sucrose in D_2O. Results are averages (five independent experiments ±SEM.

Table 3. Effect of D_2O on oxygen uptake and ADP/O, ratios by mitochondria (preparation I

D_2O (%)	Oxygen uptake (State 3 respiration) (μ atom/min/mg protein)	% Control	Oxygen uptake (State 4 respiration) (μatoms/min/mg protein)	% Control	RCI	AD]
0	0.1±0.008	100	0.04±0.016	100	3.6	1.
20	0.2±0.007*	137.6	0.05±0.019	148	3.3	0.
40	0.2±0.075	143.8	0.04±0.012	119	4.3	0.'
60	0.2±0.099	118.1	0.03±0.009	76	5.5	0.!
80	0.1±0.072	106.3	0.03±0.010	71	5.4	1.(
100	0.1±0.038	56.3	0.02±0.008	52	3.8	1.(

Mitochondrial pellet (approx. 50-60 mg) was suspended in a total volume of 1 ml of 0.25 M sucrose in H_2O and 0.02 ml of this suspension was used for determining O_2 consumption using an oxygraph. The reaction was carried out at 25°C for 5 min. Results are averages of six independent experiments ±SEM. * P<0.01.

replacement of H_2O by $D_2O(\approx 100\%$ $D_2O)$ the State 3 respiration decreased bel control values. In the absence of ADP (State 4), mitochondria from preparatio showed an increase in the oxygen uptake with increasing concentrations of D_2 upto 40% D_2O, but decreased to below control values at 60% D_2O and higher cc centrations. The respiratory control index (RCI) was found to increase wi increasing concentrations of D_2O, with mitochondria from preparation I, t maximum value being seen at 60% D_2O, (table 3), whereas at total replacement H_2O by D_2O (100%) the RCI value was not significantly different from that control. The ADP/O ratio was significantly reduced at all concentrations of D_2(except at the highest concentration ($\approx 100\%$) D_2O in the incubation medium, tl ADP/O ratio was similar to that of the control mitochondria.

With a mitochondrial preparation preincubated in D₂O (preparation II) there was a significant D_2O concentration dependent reduction in State 3 respiration (table 4). A similar trend was seen in the O_2 uptake in the absence of ADP (State 4).

Table 4. Effect of D_2O on oxygen uptake and ADP/O, with mitochondria suspended in sucrose in D_2O (preparation II).

D_2O (%)	Oxygen uptake (State 3 respiration) (μ atoms/min/mg protein)	% Control	Oxygen uptake (State 4 respiration) (μ atoms/min/mg protein)	% Control	RCI	ADP/O
0	0.08±0.002	100	0.03±0.003 ·	100	2.9	1.8
20	0.07±0.006	86	0.03±0.002	98	2.6	1.7
40	0.06±0.031	77	0.02±0.000	79	2.9	1.7
60	0.06±0.011	76	0.02±0.006	68	3.1	1.9
80	0.06±0.021	73	0.02±0.001*	60	3.5	2.0
100	0.04±0.041	50	0.02±0.040	55	2.6	1.9

Experimental details were as shown under Table 3 with the only exception that the mitochondrial suspension was also prepared in 0.25 M sucrose in D_2O. Results are averages of six independent experiments ±SEM. * P<0.01.

In both cases, with 100% D_2O in the incubation medium, the oxygen consumption decreased by 50% as compared to controls. The RCI values of preparation II were less than those of preparation I and was probably indicative of a poorer integrity of the mitochondrial membranes. It would appear that exposing the mitochondrial preparation to D_2O 15 min prior to the reaction, itself caused some structural changes, resulting in the loss of integrity to some extent. However, there did not seem to be any further damage to mitochondrial membranes with increasing concentrations of D_2O, as seen from RCI values. The ADP/O ratio was similar to control value at any concentration of D_2O.

With mitochondria from preparation II, there was a considerable decrease in State 3 respiration even in the absence of heavy water in the reaction mixture (0% D_2O), when compared to that of preparation I (table 4 and table 3). A further decrease in oxygen consumption in the presence of ADP, (State 3), was observed at increasing concentrations of D_2O. The same trend was observed with State 4 respiration.

When the data obtained from manometric and polarographic studies are compared, certain similarities as well as apparent inconsistencies are observed. With preparation I,whereas, mitochondrial respiration was consistently decreased as assessed in the manometric studies State 3 respiration showed a two way variation when determined polarographically. The P/O ratio determined in the experiments also showed a trend that was different from that of the ADP/O ratio in the oxygraph measurement. On the other hand, in the case of preparation II, the trends in oxygen consumption as well as the degree of coupling were similar in the manometric and the polarographic studies. Thus, while oxygen consumption

showed a decrease with increasing concentration of D_2O, the degree of coup
appeared unaffected.

It seems likely that these discrepancies are more apparent than real.
polarographic studies are completed within a few minutes, whereas the manome
studies require about 30 min of incubation. Thus while the former may perm
clearer distinction being drawn between the solvent effect (more time-depende
and the isotope effect (requires D_2O only at the time of reaction), the latter r
not. Thus with preparation I, which is not preincubated with D_2O,
polarographic study could be expected to give a better assessment of the isot
effect than the manometric studies, as the latter necessarily requires a long per
of incubation. An analysis of the data, bearing these considerations in mi
indicated that the inhibition of mitochondrial respiration might be a manifestat
of the solvent effect. This conclusion is in accordance with an earlier repor
observation on the rate of dehydrogenation of dideutero and tetradeut
succinates by succinoxidase (Thorn, 1951). These compounds were found to
dehydrogenated less rapidly than protosuccinate, the extent of inhibition increas
linearly with increasing concentration of deuterium. The Michaelis constant
deuterosuccinate (Thorn, 1951) was 1.5 times that for protosuccinate.

The nature of the influence of water on respiratory chain reaction can at pres
be discussed only in general terms. There seems to be universal retardation
half-times of various biological electron transfer reactions involving membra
bound electron carriers in photosynthetic bacteria and mitochondria in
presence of heavy water (Kihara and McRay, 1973). Uncoupling of oxidat
phosphorylation is essentially an isotope effect of heavy water. It would furtl
appear that where both types of effects may be operative, the former masks
latter.

To understand the possible mechanism of the uncoupling action of D_2O,
activity of mitochondrial ATPase has been studied in the presence of D_2O. T
effects of heavy water on mitochondrial ATPase are shown in table 5. D_2O is se
to enhance the basal mitochondrial ATPase activity significantly, wh
mitochondria from preparation I were used as enzyme source. On the other har
with mitochondria preincubated in D_2O (preparation II) as the enzyme sour
when the solvent effect may be expected to predominate, there was found to b
decrease in the activity of mitochondrial ATPase. Thus, the enhancement
mitochondrial ATPase activity, seems probably, due to the predominance
isotope effect. These results are in accordance with polarographic studi
However, in presence of Mg^{2+}, both with preparation I and preparation II, the
was found to be a marked inhibition in the activity of mitochondrial ATPase. Tl
is in confirmation with the earlier reported data (Margolis *et al.*, 1966). With eith
DNP alone or along with Mg^{2+}, the effect of D_2O was found to be one
inhibition, both with preparation I and preparation II.

Table 5. Effect of D₂O on mitochondrial ATPase.

Experimental conditions	Sample	ATPase activity (μmol/h/mg protein)	% Control
Basal	Control	0.34±0.083	100
	Preparation I	1.31±0.041	380
	Preparation II	0.27±0.036	79
Basal + Mg²⁺	Control	1.69±0.089	100
	Preparation I	0.29±0.005	17
	Preparation II	0.15±0.040	9
Basal + DNP	Control	5.89±0.489	100
	Preparation I	4.05±0.400	69
	Preparation II	4.83±0.748	82
Basal + DNP + Mg²⁺	Control	8.55±0.374	100
	Preparation I	4.05±0.400	47
	Preparation II	4.00±0.346	47

The incubation was carried out at 25°C for 15 min. In control samples, no heavy water was used, either during homogenisation or during incubation, wherever indicated, DNP was used at a concentration of 100 μmol. Results are averages of six independent experiments ±SEM.

It has also been separately ascertained whether some of the observed effects of heavy water would arise from the difference in the solubility of oxygen in water and heavy water. The oxygen solubility in heavy water was not found to be significantly different from that in water under the experimental conditions employed (Approved Methods for the Physical and Chemical Examination of Water published by the Institute of Water Engineers, 1953).

The mechanism of action of D₂O may be distinguished from the effect of an inhibitor such as malonate that restricts the entry of reducing equivalents into the respiratory chain. Inhibition of respiration by malonate has no effect on the P/O ratio (Van Dam *et al.*, 1966), but increases the NADPH : O ratio of the energy linked transhydrogenase (Lee and Ernster, 1966).

Our data seem to be consistent with the following hypotheses postulated by earlier investigators (Tylor and Estabrook, 1966): 1) Replacement of water by D₂O may alter the tertiary structure of the functional proteins of the respiratory chain, either by influencing the extent of their hydration by water or by inducing changes in hydrogen bonding; the modification of tertiary structure may lead to an alteration in the spatial relationship of the components of the respiratory chain and thereby reduce the efficiency of their interaction. 2) Water is directly involved in the electron transfer process itself and there exists an array of oriented water molecules between carriers of electrons, as cellular water has been shown to be more structural than pure water (Chang *et al.*, 1972) and electron transfer takes place via the effective diffusional transfer of a hydrogen atom through a water bridge.

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Fructose-1, 6-bisphosphatase in human fetal brain and liver during development

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Abstract. Activity of fructose-1,6-bisphosphatase (EC 3.1.3.11), one of the key gluconeogenic enzymes, was measured in human fetal brain and liver during development. Fructose-1,6-bisphosphatase was distributed throughout the different regions of the brain. In contrast to the partially purified enzyme from the brain, the liver enzyme was dependent on Mg^{2+} for maximal activity, EDTA, citrate, oleate and linoleate were stimulatory, whereas 5'-AMP inhibited the activity of the liver enzyme.

Keywords. Human fetus; brain and liver fructose bisphosphatase; fructose-phosphate inter-conversion; *myo*—inositol synthesis.

Introduction

During development, the human fetus depends on the mother for the supply of glucose. The glucose homeostasis of the fetus not only aids development but also contributes to extrauterine adaptation of the fetus, in which neonatal energy metabolism supported by gluconeogenesis plays a decisive function.

In order to understand the possible role of gluconeogenesis during the development of human fetus, the present investigation on the nature and properties of fructose-1,6-bisphosphatase (EC 3.1.3.11), a key gluconeogenic enzyme was undertaken. The two organs considered for this study were the fetal liver and brain. Although the brain is not ordinarily considered to be a gluconeo-genic organ, it was taken into consideration because of the unequivocal demonstration of the enzyme therein (Majumder and Eisenberg, Jr., 1977). The present communication deals with the preliminary studies on fructose bisphosphatase during development of human fetal liver and brain.

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Materials and methods

Human fetuses of different gestation periods (determined by considering the last menstrual period) were obtained from normal mothers undergoing hysterotomy and ligation in the SSKM hospital attached to the Institute of Post Graduate Medical Education and Research, Calcutta, as a part of the Medical Termination of Pregnancy Clinic. The project was cleared by the Ethical Sub-Committee of the Institute of Post Graduate Medical Education and Research. The fetuses were put on ice immediately after the operation and the liver and brain were dissected within 30 min, after the operation.

Fructose- 6-bisphosphate, sedoheptulose-1,7-bisphosphate, glucose-6-phosphate, fructose-6-phosphate, and sodium β-glycerophosphate were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Sodium salts of oleate and linoleate (Applied Science, USA) were kind gifts of Dr. Eisenberg, Jr. of the National Institutes of Health, Bethesda, USA. Partially purified fructose bisphosphate was isolated from human fetal brain of different stages of gestation following essentially the procedure of Majumder and Eisenberg, Jr. (1977) described for the enzyme from rat brain. A 25% homogenate of a portion of tissue from different parts of brain in 0.15 M KCl containing 0.01 M $NaHCO_3$ was centrifuged at 10,000 g for 30 min in a refrigerated Remi K-24 centrifuge. The supernatant was heated for 3 min at 60°C and again centrifuged at 15,000 g as before. The heated supernatant was adjusted to pH 4.8 with 5 M acetic acid and immediately centrifuged at 10,000 g. The supernatant thus obtained was adjusted to pH 7.5 (termed acid fraction), dialysed against 0.005 M Tris-HCl, pH 7.5 and used as the enzyme source.

A somewhat different procedure was adopted for the isolation and partial purification of the enzyme from human fetal liver. A 10,000 g supernatant was obtained after centrifuging a 25% 0.15 M KCl/0.01 M $NaHCO_3$ homogenate of fetal liver. This was centrifuged at 100,000 g for 1 h in a MOM (Hungary) Ultra-centrifuge. The supernatant obtained was heated for 3 minutes at 60°C and the coagulated protein centrifuged off at 20,000 g in a Remi K-24 (India) refrigerated centrifuge. The supernatant thus obtained (termed heat fraction) was dialysed against 0.005 M Tris-HCl, pH 7.5 and used as the enzyme in this study.

Assay for fructose bisphosphatase activity was carried out by the procedure of Majumder and Eisenberg, Jr. (1977) by measuring the released inorganic phosphate (Ames 1966). The standard incubation mixture contained the following (all in μmol ml); Tris-HCl buffer (pH 7.5/9.5) 100; $MgSO_4$, 5; fructose-1, 6-bisphosphate, 0.1-0.5; and enzyme, appropriate amount. It was incubated at 37°C for an hour, deproteinized by the addition of 5% trichloroacetic acid and the supernatants assayed for inorganic phosphate. Parallel sets were run using fructose-6-phosphate as substrate for determination of any non-specific phosphatase activity.

Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results and discussion

A comparison of the fructose bi-phosphatase activity at different stages of gestation revealed appreciable change in the activity in both fetal brain and liver during development (table 1). The relative enzyme activity at the neutral and

Table 1. Fructose-1,6-bisphosphatase activity of human fetal brain & liver at different gestation periods.

Gestation period (in weeks)	Body wt (in g)	Fructose bisphosphatase activity (μmol P_i liberated/ h /mg protein)			
		Brain		Liver	
		pH 7.5	pH 9.5	pH 7.5	pH 9.5
8 to 12	4.4	0.183	0.241	—	—
	8.3	0.496	0.496	0.78	—
	10.3	0.32	0.38	—	0.58
13 to 16	15.0	—	—	0.20	0.38
	15.7	0.241	0.274	—	—
	16.4	—	—	0.16	0.47
	34.3	0.132	0.164	—	—
	37.0	—	—	0.49	0.60
	38.8	—	—	0.17	0.25
	47.1	0.158	0.274	—	—
	50.0	0.158	0.174	—	—
	73.3	0.248	0.064	—	—
	103.0	0.151	0.191	—	—
	108.0	0.281	0.248	—	—
17 to 20	113.0	—	—	0.33	0.33
	172.0	0.106	0.145	—	—
	265.0	—	—	0.165	0.204
21 to 24	310.0	0.125	0.091	—	—
	392.0	0.15	0.0	0.90	0.28
	524.0	0.053	0.32	0.19	0.49
	560.0*	0.44	0.52	0.10	0.43
28 to 32	1553.0	—	—	0.56	0.57
Adult	—	1.18	0.96	—	—

The enzyme activity was determined in the 10,000 *g* supernatant as described in Methods and expressed as μmol P_i liberated per mg protein per hour after correction for the Fructose-6-Phosphatase activity, if any. Other details are in Methods.

— Not detected

alkaline range varied at different stages of development, although there was a tendency towards even distribution at the two pH values. The fructose-6-phosphatase activity of the different samples, if at all, was about 20% that of fructose bisophosphatase activity. Variation was noticed among the specific activity values for the brain and liver enzymes. However, in case of brain, the activity of the enzyme during fetal life was much lower than that of the adult organ but higher than the adult rat brain (Majumder and Eisenberg, Jr., 1977).

Table 2 describes fructose bisphosphatase activity isolated from the fetal brain of different gestation periods at different stages of purification as outlined under Methods. It was evident that, in all the specimens, except for that of 21-24 weeks

Table 2. Fructose-1,6-bisphosphatase and fructose-6-phosphatase activity during purification of human fetal brain obtained at different stages of development.

Stage of purifica- tion	Gestation period															
	8-12 weeks				13-16 weeks				17-20 weeks				21-24 weeks			
	Fru-P$_2$ase at pH		F-6-pase at pH		Fru-P$_2$ase at pH		F-6-pase at pH		Fru-P$_2$ase at pH		F-6-pase at pH		Fru-P$_2$ase at pH		F-6-pase at pH	
	7.5	9.5	7.5	9.5	7.5	9.5	7.5	9.5	7.5	9.5	7.5	9.5	7.5	9.5	7.5	9.5
10,000 g super- natant	.158	.158	0	0	.158	.190	.026	.026	.183	.222	.077	.077	.183	.181	.058	.090
Heat fraction	.664	.412	0	0	.148	.200	0	.016	.032	.280	.064	.232	.116	.174	0	.032
Acid fraction	1.32	1.32	0	0	.412	.438	0	0	.741	0	.125	.061	.174	.206	0	0

Fru-P$_2$ase and F-6-Pase activities were determined as described in Methods and expressed as µmol P$_i$ liberated per mg protein per hour. Procedures for partial purification are described in Methods.

Fru-P$_2$ase — fructose-1,6-bisphosphatase
F-6-pase — fructose-6-phosphatase.

of gestation, there was a progressive increase in the specific activity during purification. Better purification was achieved with specimens between 8-12 weeks of gestation, a stage also characterized by no detectable fructose-6-phosphatase activity. The ratio of the activities at pH 7.5 and pH 9.5 was close to 1. It may be worth mentioning here that although mammalian liver fructose bisphosphatase is reported to exhibit two activities at pH 7.5 and pH 9.5 as a result of proteolytic modification (Horecker *et al.*, 1975) the rat brain enzyme failed to show two peaks of activity over a pH range of 6 to 10 (Majumder and Eisenberg, Jr., 1977). Human fetal brain fructose bisphosphatase exhibited a similar property. Scanning of

different parts of the fetal brain e.g. cerebrum, midbrain and cerebellum showed that the enzyme was distributed in all these regions. Little variation in the distribution was noticed in samples between 8 to 24 weeks of gestation (table 3).

Table 3. Distribution of fructose-1,6-bisphosphatase activity in different regions of human fetal brain, from different stages of development.

Gestation period (in weeks)	Body weight (in g.)	Fructose-1,6-bisphosphatase activity (μmol P_i/hour/mg. protein)					
		Cerebrum		Midbrain		Cerebellum	
		pH 7.5	pH 9.5	pH 7.5	pH 9.5	pH 7.5	pH 9.5
8-12	10	0.61	0.26	0.19	0.49	—	—
13-16	45.5	0.12	0.31	0.13	0.35	0.48	0.41
	58	0.28	0.23	0.29	0.29	0.24	0.11
17-20	157	0.29	0.49	0.28	0.88	0.49	0.63
	207	0.22	0.22	0.30	0.61	0.49	0.92

10,000$\times$g supernatant from different regions of fetal brain, of different stages of gestation was used as enzyme source. Fructose-bisphosphatase activity was corrected for Fructose-6-phosphatase activity, if any.

Some properties of the fructose bisphosphatase partially purified from fetal brain (acid-fraction) and liver (heat-fraction) were studied. Substrate specificity of the enzymes from the two sources, using various sugar phosphates, such as fructose-1,6-bisphosphate, sedoheptulose-1, 7-bisphosphate, glucose-6-phosphate, fructose-6-phosphate and β-glycerophosphate (each 0.5 mM) revealed that both the enzymes were unable to hydrolyze glucose-6-P, fructose-6-phosphate and β-glycerophosphate. The brain enzyme hydrolyzed sedoheptulose-1, 7-bisphosphate to 20% of the fructose-1, 6-bisphosphate activity whereas 37% of the activity was recorded for liver enzyme with the same substrate.

Further studies revealed that while the liver enzyme was dependent on divalent cation for maximal activity, the brain enzyme had no requirement for it. Mn^{2+} at 5 mM was optimally effective and could be partially replaced by Mn^{2+} (2mM). The liver enzyme was stimulated by EDTA (0.1 mM) and inhibited by 5'-AMP (0.5 mM). Citrate (0.5 mM), oleate (0.5 mg/ml) and linoleate (0.5 mg/ml), increased the activity of the liver enzyme approximately by 2,1.5 and 2.3 fold respectively. This property was also shared by the rabbit liver enzyme (Baxter *et al.,* 1972, Carlson *et al.,* 1973). All these compounds had little effect on the brain enzyme activity.

Studies on the gluconeogenic enzymes during fetal life were mainly undertaken in human, sheep, guinea-pig and baboon (Aurricchio and Rigillo, 1960; Ballard and

Oliver, 1965; Jones and Ashton, 1972; Levitsky *et al.,* 1976). For obvious reasons, a detailed study of these enzymes in the human fetus, in particular, was lacking and subhuman primates were often chosen for this purpose (Robinson *et al.,* 1980; Sherwood *et al.,* 1980). Most of these studies dealt with determination of activities of different gluconeogenic enzymes in cell extracts from liver or kidney. In the present study we have restricted ourselves to the demonstration of the fructose bisphosphatase activity and the partial purification of the enzyme from both the organs upto a stage where it exhibits absolute specificity towards hydrolysis of fructose bisphosphate. The preliminary characterization of the liver enzyme reported here, showed that with respect to the divalent cation requirement, EDTA stimulation and 5'-AMP inhibition, it was similar to the enzyme from other mammalian sources and recently reported adult human liver enzyme (Dzugaj and Kochman, 1980).

Though not considered gluconeogenic (Scrutton and Utter, 1968), existence of fructose bisphosphatase in brain was suggested by [^{14}C]-pyruvate and [^{14}C]-glutamate incorporation studies in guinea-pig brain slices (Phillips and Coxon, 1975) and subsequently its presence in rat brain was demonstrated (Majumder and Eisenberg, Jr., 1977; Hevor and Gayet, 1978). Majumder and Eisenberg, Jr. (1976-1977) suggested that in brain, this enzyme along with the phosphofructokinase, may be regulated by fatty acids and phospholipids for controlled utilization of glucose-6-phosphate for synthesis of *myo*-inositol by the *myo*-inositol synthase reaction. Keeping this in view, a probable coupling between the two enzymes has recently been suggested (Majumder, 1981). Since during its development, the human fetal brain requires a high pool of *myo*-inositol for synthesis of polyphosphoinositides of the myelin (Eichberg and Dawson, 1965; Eichberg and Hauser, 1973), this system was thought to be ideal for this study. As preliminary to this we have demonstrated the presence of a fructose bisphosphatase in human fetal brain during development and studied some of their properties. Partial purification of *myo*-inositol synthase from rat brain was reported (Maeda and Eisenberg Jr 1980) and human fetal brain has been found to have appreciable synthase activity (Adhikari and Majumder, unpublished observation). Further studies would require a detailed characterization of the purified enzyme from both liver and brain as well as that of *myo*-inositol synthase. Work in this direction is now in progress.

Acknowledgement

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Photoperiodic effect on some enzymes and metabolites in diapausing *Antheraea mylitta* pupae and *Philosamia ricini* larvae during development

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Abstract. The variation in acid phosphatase (EC 3.1.3.2) activity in *Antheraea mylitta* was similar in all light and dark groups exposed to different photophases (LD=0:24, 24:0, 18:6, 14:10, 10:14 and 12:12 h) maintaining all along a higher activity than its alkaline counterpart. The highest activity was recorded on day 82 in LD group 10:14 h. The non-diapausing *Philosamia ricini* larvae registered highest activity in LD group 0:24 h on day 5. Alkaline phosphatase (EC 3.1.3.1) activity was low all through metamorphosis in both the lepidopterans, although significantly elevated activity was observed on day 5 in larvae of all *Philosamia ricini* LD regimens and on day 82 in *Antherae mylitta*. Photoperiodic effect on phosphorylase (EC 2.3.1.1) activity, glycogen and inorganic phosphates content have also been studied.

Exposure to LD 10:14, 14:10 and 18:6 h provoked early diapause termination in *Antheraea mylitta*. The non-diapausing *Philosamia ricini* was unaffected in moth emergence but the emerged adults of LD 24:0 and 0:24 h groups were unhealthy, small and did not mate or oviposit.

Keywords. Photoperiod; phosphomonoesterases; phosphorylase; glycogen; *Antheraea mylitta*; *Philosamia ricini*.

Introduction

Several lepidopteran insects during their life cycle undergo a phase of diapause while confronted with unfavourable environmental conditions of temperature and day length. The effect of photoperiodism on enzymic activities and metabolites concentrations in developing insects when subjected to different photophases have not been investigated. In insects the presence of a number of phosphomonesterases of undetermined specificity has been detected by employing biochemical and histochemical techniques (Drilhon and Busnel, 1945; Denuce, 1958; Faulkner, 1955). Pant and Lacy (1969) suggested that acid phosphatase participates in glycogen degradation on the assumption that the high activity of the enzyme stimulates *Corpus cardiacum* hormone which by activating phosphorylase induces glycogen degradation.

Some significant changes in amino-transferase activity, protein and total free amino acid content were observed when diapausing pupae of tasar silkworm *Antheraea mylitta* were exposed to different light and dark periods (Pant and Jaiswal, 1981). The present communication describes changes in the profile of active phophorylase, acid and alkaline phosphatases, inorganic phosphates and glycogen content in the fat body of *A. mylitta* diapausing pupa when subjected to different photoperiodic regimens of light and darkness, and compared with the changes occuring in the non-diapausing lepidopteran *Philosamia ricini* subjected to different photoperiodic exposures. *A. mylitta*, unlike *P. ricini* is a bivoltine lepdopteran undergoing a diapause for 180 days under adverse conditions of temperature while *P. ricini* is a non-diapausing multivoltine insect completing four to six cycles in a year.

Materials and methods

Larvae of *P. ricini* and diapausing cocoons of *A. mylitta* were reared and maintained under conditions as described earlier (Pant and Jaiswal, 1981). Three groups of 600 larvae of *P. ricini* at 4th instar stage were exposed to LD:24:0, 0:24 and 12:12 h. Assays were commenced after acclimatizing the insects to the experimental conditions of light-darkness exposures till they ecdysed to fifth instar stage. The light source employed in both insect experiments was 45 watt fluorescent tube.

Fat body homogenates (20% w/v) of both the insects at different developmental stages were prepared as previously described (Pant and Morris, 1969; Pant and Pandey, 1980). Three lots of six randomly selected pupae and larvae from each group were used. The strained individual and pooled homogenates of each group were assayed in triplicates and the average values were used to calculate standard deviations. Where the range was negligible no mention of any variation is made.

Acid (EC 3.1.3.2) and alkaline phosphatases (EC 3.1.3.1) were assayed as described earlier by Pant and Lacy (1969). Enzymic activity has been expressed as µg inorganic phosphate (P_i) liberated/mg protein/h. Inorganic phosphates were determined by Allen's method (1940) and expressed as mg phosphorus/g tissue fresh weight.

Phosphorylase (EC 2.4.1.1) activity was assayed by the method of Green and Stumpf (1942) as modified by Srivastava and Krishnan (1961). Enzymic activity was expressed as µg P_i/mg protein/30 min. Glycogen was isolated as described by Wiens and Gilbert, (1967) and determined by the method of Carrol *et al.* (1956) and expressed as mg glucose/g tissue fresh weight.

All estimations were made only upto day 131 since emergence commenced in one of the experimental insect groups thereafter.

Results and discussions

Light and dark period exposures of 18:6, 14:10 and 10:4 h enhanced emergence by days 45, 33 and 27, respectively; while the group exposed to continuous light (LD= 24:0 h) emerged almost normally (day 182). However, in the insect group kept totally

unexposed to light (LD=0:24 h) emergence was delayed by 21 days (table 1). In LD groups 18:6, 14:10 and 10:14 h while moth emergence percentage was 80-95% in LD groups 24:0 and 0:24 h it was 67 and 31% respectively with very high mortality rate in the latter group.

Table 1.

L : D exposure (h)	Antheraea mylitta		Philosamia ricini	
	Day of emergence	Adults % emerged	Day of emergence	% Adults emerged
12 : 12	179	98	18	90%
24 : 0	162	67	18	5%
0 : 24	200	32	18	25%
18 : 6	135	92	—	—
14 : 10	146	95	—	—
10 : 14	152	80	—	—

L : D = Light : dark.

Photoperiodic exposures appeared to have no effect on the non-diapausing *P. ricini* pupae in moth emergence. In LD 0:24 h group the pupae were shrivelled and very much shrunk in size and emergence was only 25%, while in LD 24:0 h group only 5% of the pupae emerged. The emerged adults of both groups appeared unhealthy and diminutive in size. Further, they did not mate or oviposit.

The acid and alkaline phosphatase activity of the fat body of the diapausing *A. mylitta* pupae exposed to different photoperiodic regimens varied similarly but the acid enzyme was present at a higher level all through development till emergence (figures 1Aa–Fa). The low activity present in almost all groups during days 54-68 increased on day 82,except in group LD 24:0 h (figure 1Ba) and reached the highest activity in group LD=10:14 h (figure 1 Da). Subsequently, the enzyme activity decreased and reached very low levels on day 96 in LD groups 10:14 and 14:10 h (figures 1 Da, Fa). On day 103 in LD 12:12, 24:0 and 0:24 h (figures 1 Aa, Ba, Ca) and on day 117 in LD 18:6 h (figure 1 Fa). With pupal development the activity also correspondingly increased till day 131 in all groups barring LD 10:14 and 14:10 h (figures 1 Da, Ea) where the activity got depleted.

Alkaline phosphatase on the other hand, as represented in figures 1Ab-Fb maintained a low level all through development and attained the highest activity in general, between days 82 and 96. However, in group LD 14:10 h although the enzyme revealed some activity on day 103 there was practically no activity during days 82-96.

The concentration of inorganic phosphate (figures 2Ac-Fc) varied more or less in a similar manner in all the 6 experimental insect groups. With an initial low concentration during early pupal development till day 75, they increased significantly between days 82 and 96, the maximum accumulation being in LD 0:24 and 12:12 h (figures 1Ac, Cc).
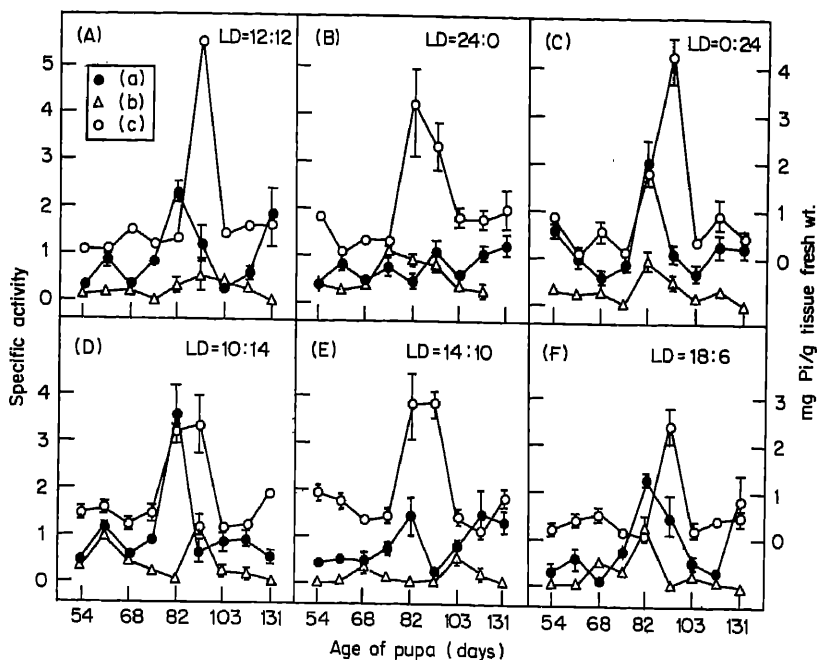
Figure 1. Alteration in the levels in acid phosphatase (●) alkaline phosphatase (Δ) and inorganic phosphates (O) in the fat body of the diapausing pupae of *A. mylitta* under different photoperiodic exposures.
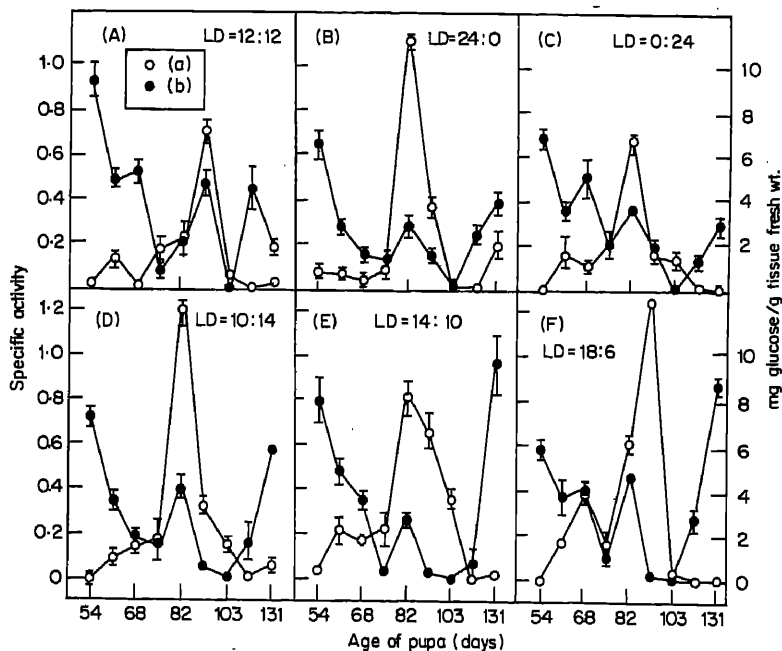
Figure 2. Phosphorylase activity (O) and glycogen (●) in the fat body of diapausing pupae of *A. mylitta* under different photoperiodic exposures.

In the non-diapausing *P. ricini* all the three exposures (LD 12:12, 24:0 and 0:24 h) revealed steady and regular increase in acid phosphatase activity each rise followed by an alternate low activity (figures 3Aa-Ca). The highest activity was recorded 0:24
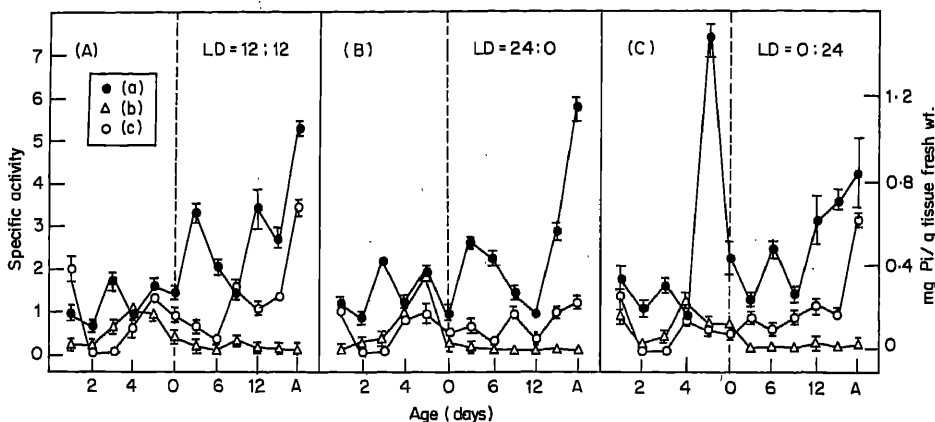
Figure 3. Changes in acid phosphatase (●), alkaline phosphtase (Δ) and inorganic phosphtates (O) in fat body of *P. ricini* under different photoperiodic exposure.

h on day 5 (figure 3 Ca) prior to commencement of spinning, the increase being seven-fold from ecdysis to fifth larval instar. However, on day zero pupa the activity was very low and interestingly, a pattern of alternating increase and decrease corresponding to that traced during larval development was observed. The net result was a two-fold increase in the activity till emergence. The LD 24:0 and 12:12 h varied in a similar manner as LD 0:24 h group without any enhanced activity. At emergence however, the activity increased several fold in both the regimens.

Alkaline phosphatase as in other insects, was maintained in all LD groups at a very low level all along larval and pupal development (figures 3 Ab-Cb). However, interestingly on day 5 prior to spinning, as in the case of acid phosphatase, the enzymic activity showed a two-fold increase. During pupal development however, the enzyme was practically inactive especially in LD 0:24 and LD 24:0 h groups (Figures 3 Cb, Bb).

Presence of appreciably high concentration of phosphates was detected in the control group in comparison with LD 0:24 and 24:0 h groups particularly on days 1, 4 and 5 in the developing larva and on pupal days 3 and 9. The newly emerged adults evinced the highest accumulation of phosphates (figures 3 Ac-Cc).

Phosphorylase activity of the diapausing *A. mylitta* pupae (figures 2 Aa-Fa) in all the groups was maintained at a very low level during early pupal period but with development the activity increased significantly, the maximum being recorded on day 82 especially in LD groups 18:6, 10:14 and 24:0 h (figures 2 Fa, Da, Ba). Thereafter, the activity decreased in all the groups and reached a minimum value on day 103 and continued at this low level till day 131.

Likewise, *P.ricini* fat body also exhibited low phosphorylase activity till day 3 in the 5th instar larva with a sudden elevation in its activity between days 4 and 5. The enzyme was maximally active in LD group 0:24 h on day 5 (figures 4 Aa-Ca).
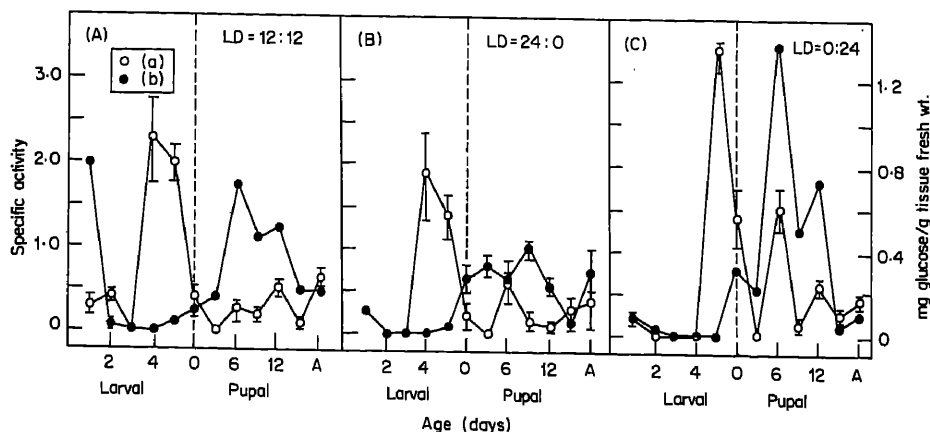
Figure 4. Variation in phosphorylase activity (O) and glycogen content (●) in the fat body of *P. ricini* under different photoperiodic exposures.

Glycogen concentration on the other hand, as anticipated appears to be governed by phosphorylase activity in both the lepidopterans. The high glycogen concentration observed in *A. mylitta* during early pupal development appeared to be utilized as noted from the gradual decrease thereof till day 75. This however, was slightly elevated on day 82 in LD groups 24:0, 0:24,10:14,14:10, 18:6 h (figures 2 Bb-Fb) and on day 96 in LD group· 12:12 h (figure 2 Ab). After declining markedly on day 103 in all experimental insect groups, glycogen steadily accumulated till day 131, the highest being was in group LD 14:10 h.

During 5th instar development the *P.ricini* fat body glycogen concentration(figures 4 Ab-Cb) varied inversely with phosphorylase activity. With commencement of spinning, glycogen accumulation increased the maximum being in the 6 day old pupa. The all dark insect group (LD 0:24 h) concentrated higher amount of glycogen than LD groups 12:12 and 24:0 h.

The higher level of acid phosphatase compared to that of alkaline phosphatase activity at all stages during metamorphosis (Pant and Lacy, 1969), in adults (Sridhara and Bhat, 1963) and during embryonic development (Pant and Sharma, 1976) and its irregular pattern throughout the pupal period (Pant and Lacy, 1969) is a common phenomenon noted in several insects. Cori and Cori (1939) suggested that the occurrence of high acid phosphatase activity in the virtual absence of its alkaline counterpart was an adaptation to an active glycogen metabolism with a dephosphory-lation in the acid range. In fact, in *P. ricini* pupae during development, a significant depletion of acid phosphatase was observed (Pant and Lacy, 1969). In addition, the present observations on the two lepidopteran insects *P.ricini* and *A.mylitta* subjectedto different photoperiodic exposure further confirm this view.

One of the major carbohydrate reserves in insects is glycogen which is stored mainly in the fat body and to some extent in muscles and other tissues, the enzyme phosphory-lase being one of the regulators of its synthesis and breakdown. This enzyme has been extensively studied by Cori and his collaborators (1943) who established its role in

glycogen synthesis. Phosphorylase breaks down glycogen to glucose and trehalose in order to provide energy for the vital processes like moulting, reproduction and flight etc. The *corpus cardiacum* hormone is believed to activate phosphorylase and regulate glycogen levels. *A. mylitta* and *P. ricini* pupal fat body recorded high glycogen content during mid-pupal stage which in all probability is the remnant of the rich glycogen reserves accumulated towards the end of the voraciously feeding larval period. This high level declines steadily during pupal development when histolysis proceeds at a higher rate than histogenesis and practically disappears in the newly emerged adults. Bade and Wyatt's (1962) findings in *Cecropia* coupled with Pant and Lacy's (1969) for *P. ricini* further support the present observation.

With a concurrent depletion in glycogen content both the lepidopterans revealed high acid phosphatase activity. Since degradation of glycogen is believed to be under the hormonal control of *corpus cardiacum* (Steele, 1961) it is not unlikely that the high acid phosphtase activity observed in the developing pupa stimulates the *corpus cardiacum* hormone which in its turn enhances the phosphorylase activity resulting in the degradation of glycogen.

In both the lepidopterans, despite the steady low maintenance of alkaline phosphtase activity all through metamorphosis, a significant increased activity thereof was observed on day 5 in the pupating larva of *P. ricini* suggesting its possible role in the hormonal control during moulting. Likewise *A. mylitta* pupa also exhibited an increased activity on day 82 at mid-pupal stage.

Diapausing *A. mylitta* pupae when exposed to various photoperiodic regimens acid and alkaline phosphatase as well as phosphorylase activity became highly predominant in LD groups 18:6, 14:10 and 10:14 h. Since light indirectly influences enzymic activities in insect tissues and direct exposure to it regulates the synthesis and release of their neurohormones (Danilevskii, 1965) the various metabolic processes also get stimulated. Thus insects exposed to longer light period especially the LD groups 18:6 h exhibited high enzymic activity while the reverse was true in the groups exposed to longer dark periods (LD 0:24).

The diverse behaviour of the two insects belonging to the same order lepidoptera when exposed to different photophases during growth and development makes one wonder whether this diversity within the same order is also a biochemical characteristic of the class insecta.

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Inhibition of *in-vitro* amino acid incorporation by the carcinogen N-methyl N'-nitro N-nitrosoguanidine

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Abstract. The addition of the carcinogen, N-methyl N'-nitro N-nitrosoguanidine, to a cell-free system consisting of purified polysome and 'pH 5 enzyme' fraction resulted in a marked inhibition of incorporation of (^{14}C)-leucine into polypeptides. The extent of inhibition was remarkably high if the cell-free system contained limiting amount of 'pH 5 enzyme' fraction. Under this condition, the rate of inhibition was dependent on the concentration of carcinogen. Some component present in the 'pH 5 enzyme' fraction was inferred to be the susceptible factor, since the inhibition at low concentration of carcinogen could be reversed by increasing the amount of this fraction in the polysomal system. It was ascertained that tRNA was the primary target of carcinogenic action. Evidence suggested that functions attributed to tRNA such as aminoacylation and ribosomal transfer were both affected in a characteristic way by the action of the carcinogenic N-nitroso compound.

Keywords. N-methyl N'-nitro N-nitrosoguanidine; carcinogen; amino acid incorporation; tRNA.

Introduction

A number of studies indicate that a variety of carcinogenic N-nitroso compounds lead to marked impairment of *in-vivo* liver protein synthesis (Magee, 1958; Emmelot *et al.,* 1962; Magee and Barnes, 1967; Stewart and Magee, 1972; Arnold and Alonoso, 1973; Kleihues and Magee, 1973; Hradec and Kolar, 1974; Chu and Nirvish, 1977). A few studies also reveal that administration of certain N-nitroso compounds to rats decreases the *in-vitro* amino acid incorporating activity of their cell-free liver preparations (Hutlin *et al.,* 1960, Abakumova *et al.,* 1974). This inhibition, reflecting the acute effect of N-nitroso compounds, is perhaps dependent on their prior metabolism which is a necessary step for carcinogenic activity (Magee *et al.,* 1975; Lijinsky, 1976).

Our aim is to examine the role of chemical carcinogens at translational level and its relevance to carcinogenesis. N-methyl N'-nitro N-nitrosoguanidine (MNNG), a member of the N-nitroso group, is one useful compound for this purpose since it does not need metabolic activation for its action (Sugimura and Kwachi, 1973). Its mutagenic (Mandel and Greenberg, 1960) and carcinogenic (Druckery *et al.,* 1966;

Schoental, 1966; Sugimura *et al.,* 1966) activities have been well established. Like other N-nitroso compounds, MNNG has been shown to inhibit the synthesis of macromolecules such as DNA, RNA and protein in bacterial cells (Cerda-Olmedo and Hanawalt, 1967) as well as in mammalian cells (Anderson and Burdon, 1970). Impairment of function of the individual macromolecule modified by MNNG has also been documented (Chandra *et al.,* 1967; Drahovsky and Wacker, 1975; Bagewadikar and Bhattacharya, 1977).

Being a direct acting carcinogen, its effect on several parameters of protein synthesis can be studied in *in-vitro* model system. Our preliminary observations in this direction showed that addition of low concentration of MNNG resulted in a marked inhibition of amino acid incorporation in a cell-free system containing limiting but not saturating level of S-30 fraction derived from rat liver (Bagewadikar and Bhattacharya, 1979). The present study has been initiated with a view to identifying the limiting factor(s) of the subcellular components which is susceptible to inhibition.

Materials and methods

Chemicals

L-amino acids were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. L-(U-^{14}C)-leucine (Sp. act. 198 mCi mmole^{-1}) was a product from Isotope Division, Bhabha Atomic Research Centre, Bombay. N-methyl N'-nitro N-nitrosoguanidine was obtained from Fluka AG, Buchs SG, Switzerland. All other chemicals were of Analytical grade.

Buffers

Buffer A contained 0.2 M sucrose, 0.1 M NH$_4$Cl, 5 mM Mg-acetate, 1 mM dithiothreitol, 0.25 mM EDTA, and 0.02 M tris-HCl, pH 7.5. Buffer B had the following composition: 0.3 M sucrose, 5 mM Mg-acetate, 1 mM dithiothreitol, 0.25 mM EDTA and 0.01 M tris-HCl, pH 7.5.

Animal tissues

Male Wistar rats weighing about 150 g were used. These were sacrificed by decapitation and livers were removed quickly, washed and rinsed in ice-cold buffer A. All subsequent operations were carried out at 4°C.

Liver polysomes

The method of Flavey and Staehlin (1970) was used with suitable modification. A 40% (wt/vol) homogenate of livers was prepared in buffer A using a glass-teflon homogenizer. The homogenate was centrifuged for 10 min at 30,000 g, the upper two-thirds of the post-mitochondrial supernatant fluid was removed and treated with sodium deoxycholate at a final concentration of 1.3%. Following this treatment the solution was layered over a discontinuous gradient consisting of 1 M and 0.5 M sucrose solutions prepared in buffer A without sucrose. The gradient was centrifuged for 3 h at 105,000 g, the pellets were quickly rinsed with distilled water and then dissolved in buffer A without sucrose at a concentration of about 100 A$_{260\,nm}$ unit ml^{-1}. The polysomes thus obtained were stored in small portions at −80°C.

Liver 'pH 5 enzyme' fraction

The rat liver pH 5 enzyme fraction was prepared according to Falvey and Staehlin (1970).

Incorporation of (^{14}C)-leucine into polysomal protein

The experiment was carried out using certain modifications of the methods employed by Griffin *et al.* (1964) and Falvey and Staehlin (1970). The incubation mixture (200 µl) contained tris-HCl, pH 7.5, 4 µmol; NH$_4$Cl, 30 µmol; Mg-acetate, 0.8 µmol; dithiothreitol, 0.2 µmol; ATP, 0.2 µmol; GTP, 0.08 µmol; creatine phosphate, 1 µmol; creatine phosphokinase, 10 µg; 1-(U-^{14}C)-leucine, 0.5 µCi; polysome, 6 A$_{260}$ nm units; 'pH 5 enzyme' protein, as specified; and 19 non-labelled amino acids, 5 n mol each. When required, MNNG in distilled water was added to give a final concentration as specified. The reaction mixture was incubated for 30 min at 37°C. Aliquots of 50 µl were then spotted on Whatman No. 3 MM filter paper discs (24 mm) which were washed several times with cold 10% trichloroacetic acid (TCA). The discs were then put in 10% TCA and heated to 90°C for 10 min. After a further wash with cold 5% TCA and two washes with ethanol: ether (1:1) and ether, the discs were air dried and the radioactivity was determined.

Analytical procedure

Protein was determined by the method of Lowry *et al.*, (1951). RNA was estimated from A$_{260}$nm using A$_{1\,cm}^{1\%}$ value of 230. The radioactivity of the samples were determined in Bray's solution using a Beckman model LS-100 liquid scintillation spectrometer.

Results

In our standard assay maximum incorporation of (^{14}C)-leucine into polysomal protein (8.7×10^{4}cpm mg^{-1} RNA) was observed when the reaction mixture contained 6 A$_{260}$nm unit of polysome and 1 mg of 'pH 5 enzyme' protein. These concentrations were considered to be optimum. The pH 5 supernatant was not required in our study since all the soluble factors were either bound to polysomes or were present in the 'pH 5 enzyme' fraction. At limiting concentration of either 'pH 5 enzymes' or polysomes, the incorporation was considerably reduced. Since we were interested in identifying the factor(s) susceptible to inhibition by MNNG, most of our initial studies were conducted with limiting concentration of either one or the other component required for *in-vitro* amino acid incorporation.

Table 1 shows the inhibition by MNNG of (^{14}C)-leucine incorporation into polysomal protein under different conditions. Under optimum condition, i.e., without any limiting factor in the reaction mixture, significant inhibition was observed only at a higher concentration of MNNG. When the 'pH 5 enzyme' fraction was limiting (0.2 mg protein), however, significant level of inhibition was apparent even at a lower concentration of MNNG. At optimum concentrations of both polysomes and 'pH 5 enzymes' or at limiting polysome concentration (1 A$_{260}$nm unit), the magnitude of inhibition, as compared to that at limiting 'pH 5 enzymes' concentration, was much less with 0.05 mM or with 0.2 mM and relatively

Table 1. Inhibition of incorporation of (^{14}C)-leucine by MNNG.

Limiting factor	Percent inhibition at MNNG concentration (mM)		
	0.05	0.2	0.5
None	16	19	55
pH 5 enzyme	34	57	85
Polysome	19	33	75

Incubation mixture without any limiting factor contained 6 A_{260}nm unit polysome and 1.0 mg 'pH 5 enzyme' protein. Limiting concentration of 'pH 5 enzyme' was 0.2 mg protein and that of polysome was 1.0 A_{260}nm unit. Other conditions are as described in the text.

less with 0.5 mM MNNG. In other words, it required more MNNG to achieve 50% inhibition under optimum or limiting polysome conditions than under limiting 'pH 5 enzyme' condition. The inhibition of incorporation of (^{14}C)-leucine into polysomal protein under limiting 'pH 5 enzyme' condition was dose dependent, showing lower incorporation with increasing concentrations of added MNNG.

These results are shown in figure 1. Preincubation of 'pH 5 enzyme' fraction with 0.5 mM MNNG completely inhibited its ability to mediate (^{14}C)-leucine
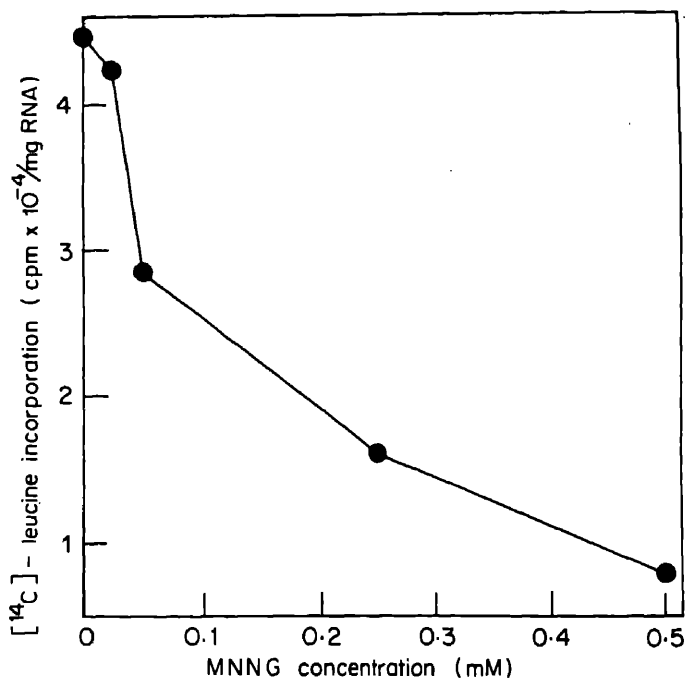
Figure 1. Effect of different concentrations of MNNG on the incorporation of (^{14}C)-leucine into polysomal protein.

Each incubation mixture contained 0.25 mg 'pH 5 enzyme' protein (limiting) and 6 A_{260}nm unit polysome. Other conditions are as described in the text.

incorporation using normal polysomes. The polysomes, on the other hand, retained more than 50% of the activity after similar treatment.

Analogous to our previous results with S-30 fraction (Bagewadikar and Bhattacharya, 1979), we have made a similar observation with the 'pH 5 enzyme' fraction. The extent of inhibition at a given concentration of MNNG was found to be inversely proportional to the amount of 'pH 5 enzyme' fraction in the assay medium. Thus the inhibition was maximum when (^{14}C)-leucine incorporation was measured with a low amount of 'pH 5 enzyme' protein. At 0.2 mM MNNG, with 0.2 mg 'pH 5 enzyme' protein, where the inhibition of (^{14}C)-leucine incorporation was to the extent of 55%, a near complete reversal of activity (92% of control) could be achieved when the amount of 'pH 5 enzyme' protein was increased to a saturating level (1.6 mg protein).These results are depicted in figure 2. Under the condition of limiting amount of 'pH 5 enzyme' protein (0.2 mg), addition of exogenous tRNA, a
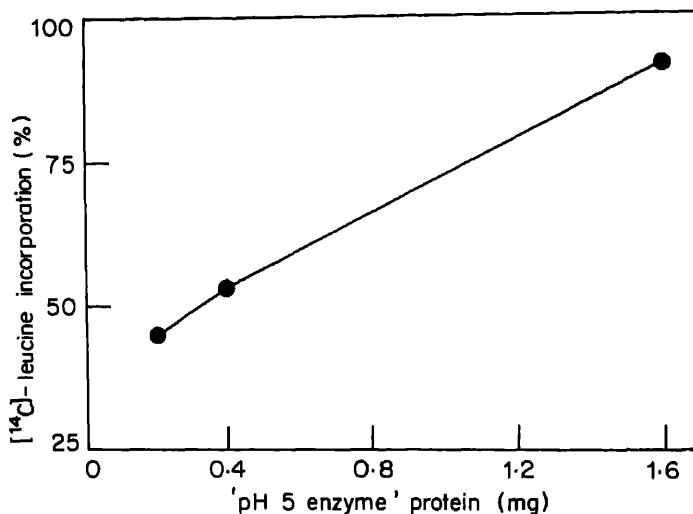
Figure 2. Reversal of inhibition by MNNG of incorporation of (^{14}C)-leucine with increasing concentrations of 'pH 5 enzyme' protein.

Each incubation mixture contained 6 A_{260nm} unit polysome and varying concentrations of 'pH 5 enzyme' protein. The concentration of MNNG was 0.2 mM. Other conditions are same as described in the text.

component of 'pH 5 enzyme' fraction, also partially reversed the inhibition induced by 0.25 mM MNNG (table 2). In this case the inhibition of 73% was brought down to a level of 21%, affording protection by 0.4 A_{260nm} unit of tRNA to the extent of 52%. Addition of more tRNA did not result in further reversal, and lower concentration was not effective.

In order to find out if the inhibition was at the level of transfer of aminoacyl-tRNA to ribosome, we conducted the following experiment. The 'pH 5 enzyme' fraction (0.8 mg protein) was incubated for 10 min at 37°C with (^{14}C)-leucine, non-labelled amino acids and other co-factors. This was performed to allow the aminoacylation reaction to be completed. After this reaction, more co-factors

Table 2. Reversal of MNNG inhibition of incorporation of (^{14}C)-leucine by exogenous tRNA

tRNA added (A_{260nm} unit)	Inhibition (%)
None	73
0.4	21

Incubation mixture contained 0.2 mg 'pH 5 enzyme' protein and 6 A_{260nm} unit polysome. MNNG concentration was 0.25 mM. Other conditions are as described in the text.

were added followed by addition of 6 A_{260nm} unit of polysomes and the mixture was inbutated at 37°C in the presence of 0.5 mM MNNG. Control sample was identical but had no MNNG. Incorporation of (^{14}C)-leucine was then measured at intervals. The results as presented in figure 3 showed that the incorporation of (^{14}C)-leucyl-tRNA preformed in the 'pH 5 enzyme' fraction proceeded well under normal experimental condition, while it was strongly inhibited in the presence of MNNG.
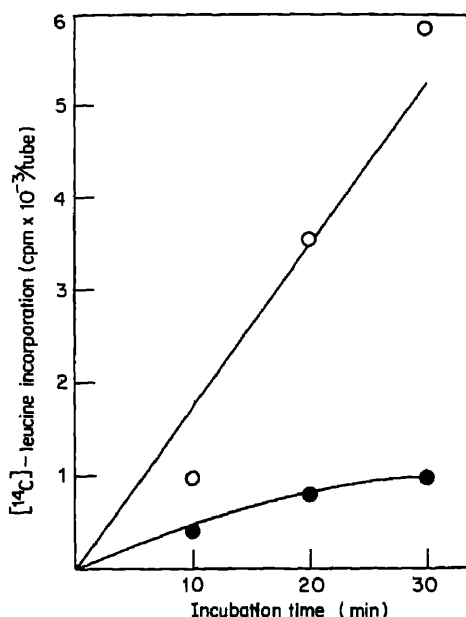
Figure 3. Effect of MNNG on the incorporation in the polysomal protein of (^{14}C)-leucyl-tRNA preformed in the 'pH 5 enzyme' fraction. Conditions are as described in the text. O, no MNNG; ●, in presence of 0.5 mM MNNG.

Discussion

Our results clearly show that MNNG, in a dose-dependent fashion, inhibits incorporation of (^{14}C)-leucine in an *in-vitro* protein synthesizing system utilizing rat

liver polysome and 'pH 5 enzyme' fraction.   The inhibition is markedly significant at a limiting concentration of 'pH 5 enzyme' protein but not at limiting concentration of polysomes or when both the components are present in optimum concentrations.   Further, the inhibition of incorporation of (^{14}C)-leucine into polysomal protein at a low concentration of MNNG is gradually reversed with the system being increasingly saturated with respect to 'pH 5 enzyme' protein.   The 'pH 5 enzyme' fraction, in contrast to polysomal fraction, is also more susceptible to inactivation when preincubated with MNNG.   These evidences as well as our earlier results (Bagewadikar and Bhattacharya, 1979) suggest that the limiting factor(s) susceptible to inhibition is present in one or more components of the 'pH 5 enzyme' fraction.   The main components involved in protein synthesis of this fraction are aminoacyl-tRNA synthetases and tRNA molecules.   In as much as the amino-acyl-tRNA synthetases are not affected by MNNG (Bagewadikar and Bhattacharya, 1977), it is reasonable to infer that the tRNA molecules are susceptible to attack by MNNG.   Our earlier observations have revealed that tRNA has great propensity for modification by MNNG (Bagewadikar and Bhattacharya, 1977).   Similar observations have been noted with other carcinogens (Farber and Magee, 1960; Magee and Farber, 1962; Weinstein, 1971; Blobstein, *et al.,* 1975; Gurtoo and Dave, 1975; Pietropaolo and Weinstein, 1975; Daoud and Griffin, 1976).   We have also demonstrated here that, under suitable condition, addition of exogenous tRNA to the *in-vitro* amino acid incorporation system can reverse the inhibitory effect of MNNG.   All these evidences indicate that MNNG as a carcinogen alters the characteristics of tRNA, through certain modification, in such a way that its ability to function normally in protein synthesis is impaired.

Having fairly etstablished that tRNA is the primary target of attack by MNNG, it is necessary to find out at what level of protein synthesis MNNG acts.   It appears likely that the aminoacylation step is primarily affected. Clear evidence in support of this has been obtained earlier (Bagewadikar and Bhattacharya,1977).In addition, it is also seen from the results presented in figure 3 that the transfer of aminoacyl-tRNA to ribosomes is prevented in the presence of MNNG. This experiment, however, is of preliminary nature.   We need more proof in this respect by studying the factor-dependent transfer of (^{14}C)-leucyl-tRNA to ribosome.

Various studies have pointed to multiple mode of action of chemical carcinogens in protein synthesis.   Grab *et al.* (1979) showed that methylazoxymethanol, a methylating carcinogen, inhibited protein synthesis in rat liver.   The site of its action was shown to be at the polysome level, and that both free and membrane bound polysomes were affected.   Dissociation of polysome was found to be the primary mechanism by which several different carcinogens inhibited protein synthesis (Sidransky *et al.,* 1977; Murthy and Verney, 1977; Sidransky and Verney, 1978).   Other effects involving different components were also observed (Hutlin, 1960; Terawaki and Greenberg, 1965; Craddock, 1969).   It is evident from our observations that the inhibition of protein synthesis by MNNG is mediated through modification of tRNA molecules, impairing both aminoacylation and transfer steps.   This modification involves hypermethylation (Bagewadikar and Bhattacharya,1977),since MNNG imparts its biological action through methylation (Sugimura and Kwachi, 1973).

The modification of tRNA may be related to suppressed protein synthesis, but its relevance to carcinogensis cannot easily be established. It can be viewed, however, that altered tRNA may lead to a functionally altered protein which otherwise may participate in controlling the perpetual growth of cells.

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Kinetic characterization of rat liver nuclear lysozyme

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Abstract. The kinetic properties of rat liver nuclear lysozyme, earlier purified to homogeneity in our laboratory, have been studied. The enzyme was found to be maximally active in the pH range 4.2 to 5.4 in 0.02 M buffer. Its K_m was found to be 333 mg/litre. It was heat sensitive even in the acidic pH range. The enzyme exhibited tissue specific differences when compared with the rat kidney nuclear lysozyme.

Keywords. Nuclear lysozyme; characterization; tissue specific differences; pH optimum; K_m value.

Introduction

Recently it was shown that multiple forms of rat liver lysozyme were located in the subcellular organelles (Sidhan and Gurnani, 1981). However, only the nuclear enzyme could be purified to electrophoretic homogeneity (Sidhan and Gurnani, 1981). Further, it was shown that rat liver nuclear lysozyme differed from rat kidney nuclear lysozyme not only with respect to its chemical properties but also with respect to its latency. In liver nuclei, lysozyme was found complexed with a protein inhibitor whereas this inhibitor was not present in the nuclear fraction of the kidney homogenate (Sidhan *et al.*, 1976). Therefore, it was of interest to examine whether the liver nuclear enzyme was kinetically different from kidney nuclear lysozyme.

Materials and methods

Glucosamine was obtained from Sigma Chemical Company, St. Louis, Missouri, USA; N-acetyl Glucosamine was the product of Kochlight Laboratories, England; Analar grade urea and guanidine hydrochloride were from Fluka (Switzerland); and EDTA was from BDH England.

The enzyme was purified (Sidhan and Gurnani, 1981; 1978) and assayed for activity (Raghunathan and Gurnani, 1971). The heat stability of the enzyme was determined by heating it in 0.2 M acetic acid for 5 min at different temperatures in the range 37°C to 80°C.

The effect of inhibitors, denaturing agents, salts etc., was examined by incorporating these agents in the buffer used for the preparation of the substrate or cell suspension.

Results

The activity of the enzyme was linearly dependent on protein concentration (30-70 µg), buffer concentration (0.0-0.02 M), incubation time of the assay (0.0-30 min) and temperature (25°C-37°C). The K_m of the enzyme calculated from a Lineweaver-Burk plot was 333 mg/litre.

The pH optimum of the enzyme was checked using different buffer systems as shown in figure 1. The enzyme attained maximum activity at pH 5.4 in phosphate
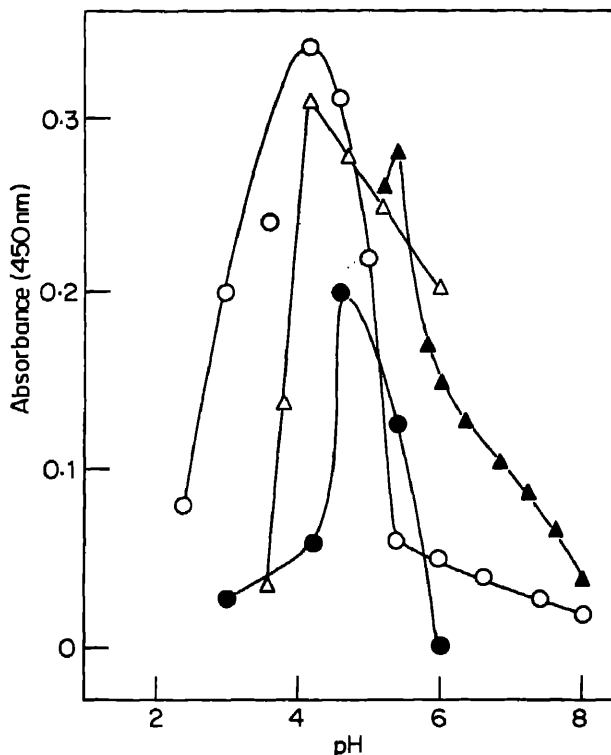
Figure 1. pH-activity profile of the rat liver nuclear lysozyme in different buffers. In each case the activity was measured at different pH values keeping the same buffer concentration (0.02 M) for 10 min at 37°C. The enzyme concentration used is indicated in parenthesis.

Sodium phosphate buffer, (76 µg, ▲);   Sodium acetate buffer, (80 µg, Δ); and
Sodium phosphate-citric acid buffer, (78 µg, O);   Sodium citrate-citric acid buffer, (78 µg, ●).

buffer. With acetate buffer and phosphate-citrate buffer, it showed a pH optimum of 4.2. Using sodium citrate-citric acid buffer, its maximum activity was observed at pH 4.4. When the effect of buffer concentration was examined at pH 5.4, it gave maximum activity at a buffer concentration of 0.02 M.

The optimum temperature required for the maximum activity was 37°C. With further increase in temperature, the activity of the enzyme decreased. At 80°C

only 25% of its original activity remained. The enzyme was found to be heat sensitive under acidic as well as alkaline conditions. In acidic conditions (see experimental section) at 80°C, it showed only 20% activity as compared to that observed at 37° whereas under alkaline conditions, it was inactivated completely within 2 min (Sidhan and Gurnani, 1978).

Glucosamine was a more potent inhibitor of the liver nuclear enzyme than N-acetyl-glucosamine (figure 2). At a concentration of 0.05 M, inhibition due to N-acetyl-glucosamine was 25 per cent, whereas that with Glucosamine was 80 per cent. Since Glucosamine was used in its hydrochloride form, the effect of
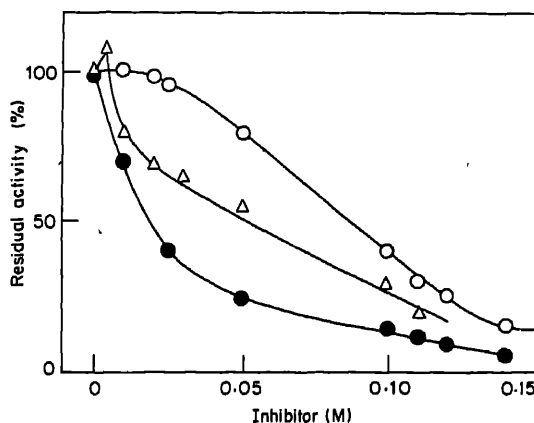
Figure 2. The effect of inhibitors on the activity of rat liver nuclear lysozyme. The assay was carried out at 37°C for 10 min in 0.02 M sodium phosphate buffer, pH 5.4 using 75 µg enzyme. NAGA (O); GA(●); NaCl, (Δ).

different concentrations of sodium chloride, on the activity of the enzyme was checked to eliminate the possibility of inhibition due to the salt. It is quite clear from the results shown in figure 2 that apart from inhibition due to the salt, there was additional inhibition (25%) caused by glucosamine, at a corresponding molar concentration.

Protein denaturing agents like urea and guanidine-HCl were also studied for their denaturing action on liver nuclear lysozyme. Guanidine-HCl, at lower concentration (0.01 M) produced a slight activation. With further increase in concentration, the activity decreased progressively. Complete inactivation was observed at 0.4 M guanidine-HCl (figure 3a). With urea, 70 per cent inhibition was observed at a concentration of 3 M (figure 3b).

Discussion

The kinetic properties of the homogeneous rat liver nuclear lysozyme showed some similarities as well as some differences when compared to the kidney nuclear enzyme. The K_m of the liver nuclear lysozyme was high having a value of 333 mg/litre as compared to that of kidney nuclear enzyme which was 200 mg/litre (Raghunathan and Gurnani, 1975).
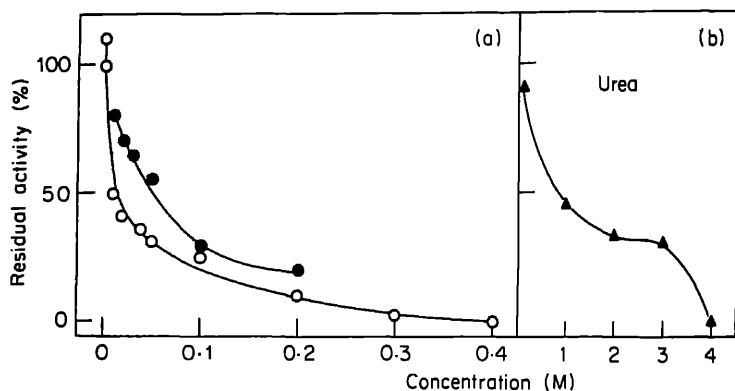
Figure 3a. Effect of guanidine-HCl and NaCl on the activity of rat liver nuclear lysozyme. Guanidine-HCl and NaCl were incorporated along with the substrate in the assay system containing 0.02 M sodium phosphate buffer pH 5.4, enzyme 75 μg, incubated at 37°C for 10 min.
Guanidine-HCl, (O); NaCl (●).

Figure 3b. Effect of urea on the activity of rat liver nuclear lysozyme.

The pH optima and the effect of buffer molarity on the activity of the liver nuclear lysozyme also differed from those of the kidney nuclear lysozyme (pH 6.2 and 9.2 in 0.06 M), the former was maximally active at low buffer molarity (0.02 M), was active in the acidic pH range 4.2 and 5.4 and did not show a second pH optimum in the alkaline region (Raghunathan and Gurnani, 1971; 1972). There was a slight shift in the pH optimum with different buffer systems suggesting the influence of ionic species in the buffer on the activity. The liver nuclear lysozyme also shared some of these features with fish, T_4 and papaya lysozymes with respect to the requirement of low buffer concentration and low pH for its optimum activity (Sankaran and Gurnani, 1972; Jenson and Kleppe, 1972; Haward and Glazer, 1969).

Like lysozymes from other sources (Imoto *et al.*, 1972), liver nuclear lysozyme was maximally active at 37°C and was rapidly inactivated on heating and differed from the kidney nuclear lysozyme in being heat sensitive under acidic conditions.

The response of the liver nuclear lysozyme to the inhibitors, glucosamine and N-acetyl glucosamine, the known competitive inhibitors of lysozyme, was comparable to their effect on kidney nuclear lysozyme (Raghunathan and Gurnani, 1975). However, in the case of liver nuclear lysozyme, the former was more inhibitory than the latter; the opposite was true with the kidney nuclear lysozyme. At low concentrations, sodium chloride had an activating effect on the liver nuclear enzyme, but with increase in concentration there was progressive inhibition, similar to that seen in the case of the kidney nuclear lysozyme.

The effect of denaturing agents on the activity of the liver nuclear lysozyme appeared to suggest that guanidine hydrochloride was a more powerful denaturing agent than urea, as was earlier observed in the case of the kidney nuclear lysozyme (Raghunathan and Gurnani, 1975). The loss of activity of hen egg white lysozyme in a low concentration of guanidine-hydrochloride was shown to be due to the effect of ionic strength (Altekar and Gurnani, 1972). In this respect, the liver nuclear lysozyme responded like the kidney nuclear enzyme and hen egg white lysozyme.

Thus the investigation of the kinetics of rat liver nuclear lysozyme further confirms the tissue specific differences between rat liver nuclear lysozyme and rat kidney nuclear lysozyme. The differences in their physiochemical properties have been reported elsewhere (Sidhan and Gurnani, 1978).

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Isolation and characterization of the 7S protein from glanded cottonseed *(Gossypium herbacium)*

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Abstract. The major protein from glanded cottonseed has been isolated in a homogeneous form. Its $S_{20,w}$ value at 1% protein concentration is 6S in 1 M NaCl solution. It contains 1% carbohydrate and is free from phosphorus, gossypol (bound or free) and nucleic acid impurities. It consists of atleast seven non-identical subunits. The protein has an ultraviolet absorption maximum at 278 nm and fluorescence excitation and emission maxima at 280 nm and 325 nm respectively. Optical rotatory dispersion and circular dichroism measurements indicate that the protein consists predominantly of β-structure and random coil. The observed near-ultraviolet circular dichroic bands can be attributed to tyrosine, phenylalanine and tryptophan residues of the protein.

Keywords. Isolation; characterization; cottonseed protein; 7S protein; gossypol.

Introduction

Cottonseed proteins consist of three protein fractions, which are designated as 2S, 7S and 12S protein based on their sedimentation coefficient (Martinez *et al.*, 1970). The 7S protein constitutes about 50% of the total proteins and is the major protein component (Martinez *et al.*, 1970). Many methods have been reported for the isolation of the 7S protein (Karon *et al.*, 1950; Rossi-Fanelli *et al.*, 1964; Ibragimov *et al.*, 1969; Phillips and Martinez, 1973; Youle and Huang, 1979). Some of its physico-chemical properties have also been reported (Rossi-Fanelli *et al.*, 1964; Ibragimov *et al.*, 1969; Phillips and Martinez, 1973; Youle and Huang, 1979; Zarins and Martinez, 1974). However, no information is available on the conformation of the protein. In this communication a method is described to isolate the 7S protein in a homogeneous form and the spectral and conformational properties of the protein are reported. This forms a part of our investigation on the conformation of isolated oilseed protein fractions and the effect of denaturants on them.

Materials and methods

Materials

Cottonseeds of the variety Jayadhar (*Gossypium herbacium*) were obtained from the Agricultural Research Station, University of Agricultural Sciences, Dharwar, Karnataka. Sepharose 6B-100 (40-210 µ) from Pharmacia Fine Chemicals (Sweden), DEAE-cellulose (coarse mesh of 0.80 meq/g exchange capacity), tetrasodium pyrophosphate, bovine serum albumin, egg albumin, pepsin, β-lacto-globulin and ribonuclease A from Sigma Chemical Co., St. Louis, Missouri, USA, Coomassie brilliant blue from Schwarz-Mann, New York, USA, acrylamide from E. Merck, Munich, West Germany, bis-acrylamide from Koch-Light Laboratories Ltd., Colnbrook, England, N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED) and β-mercaptoethanol from Fluka, Switzerland were used. All other chemicals used were of Reagent grade.

Isolation of the 7S protein

Cottonseeds were flaked in a Kvarnmaskiner (Type 5) flaking machine, dried in air, sieved to remove the hulls, defatted with n-hexane at room temperature (~30°C) and the solvent removed by aeration. The flakes free of the solvent were milled in an apex comminuting mill to a mesh size of 60 (B 3325). Moisture and protein content of the meal were 9.5% and 48%, respectively.

Ten grams of the defatted cottonseed flour were stirred with 100 ml of 1 M NaCl solution for 1 h. The insoluble residue was separated by centrifugation at 3330 *g* for 30 min and the clear supernatant dialysed against distilled water for 6 h. The water insoluble proteins in the dialysis tubing were separated by centrifugation at 2030 *g* for 30 min and the supernatant discarded. The precipitate was washed 2-3 times with small portions of distilled water, dissolved in 10-15 ml of 0.05 M tetrasodium pyrophosphate-HCl buffer of pH 7.8 (buffer A) and dialysed against the same buffer. The clear protein solution was loaded on DEAE-cellulose column (2.5×25 cm) equilibrated with buffer A. Protein and gossypol were monitored by measuring the absorbance at 280 nm and 358 nm respectively. The unadsorbed proteins were eluted with 300 ml of the same buffer and rejected. The adsorbed protein(s) was eluted with buffer containing 0.1 M NaCl. The peak fractions were pooled and the protein precipitated with 40% ammonium sulphate (w/v). The precipitate was dissolved in buffer A and dialysed against the same buffer. The dialysate was rechromatographed with a continuous gradient of NaCl (0 to 0.6 M) on DEAE-cellulose column (2×16 cm) equilibrated with buffer A. The unadsorbed protein was rejected. The peak fractions eluting at 0.1 M NaCl were pooled and precipitated with 40% ammonium sulphate (w/v). The precipitate separated by centrifugation was dissolved in the required solvent and dialysed against the same.

Disc electrophoresis

Disc electrophoresis with polyacrylamide gels was carried out according to the method of Davis (1964), using 10% gels in 0.025 M tris-glycine buffer of pH 8.3. Gels were stained with 0.25% Coomassie brilliant blue and destained in water containing 7.5% acetic acid and 5% methanol (Weber and Osborn, 1968).

Sedimentation velocity: Sedimentation velocity measurements were made at 59,780 rpm at 20°C in a Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics and Rotor Temperature Indicator and Control (RTIC) unit. A 12-mm single sector duraluminum cell was used with 1% protein solution in 1 M NaCl. $S_{20,w}$ value was calculated by the standard procedure (Schachman, 1959).

Gel filtration: Sepharose 6B-100 equilibrated with 1 M NaCl solution was packed into a column, 2×100 cm. Elution of the protein was done with the same solvent. Fractions (3 ml) were collected and protein was monitored by measuring the absorbance at 280 nm. Absorbance at 358 nm, which is a measure of gossypol concentration (both bound and free) was also measured.

Protein concentration was estimated by measuring the absorbance of the solution at 280 nm. For the 7S protein a value of 6.0 for $E^{1\%}_{1\,cm}$ at 280 nm was used. This value was obtained by measuring the absorbance of a series of solutions whose concentrations were determined by the microKjeldahl procedure for the estimation of nitrogen. A value of 6.25 was used for converting nitrogen to protein.

Absorption spectrum: Ultraviolet (UV) absorption spectrum of protein in buffer A was recorded at room temperature ($\sim$30°C) in a Perkin-Elmer double beam recording spectrophotometer 124 in the range 240 to 370 nm.

Fluorescence spectrum of the protein was measured in a Perkin-Elmer fluorescence spectrophotometer, Model 203, at 25°C. The emission spectrum was measured in the range of 300-400 nm after excitation at 280 nm. The excitation spectrum was measured in the range of 240 to 300 nm after fixing the emission maximum at 325 nm. Protein solutions (0.01%) were used for these measurements.

Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra: These measurements were made on a JASCO-J20C automatic recording spectropolarimeter calibrated with d-10 camphor sulphonic acid. Quartz cells (1 cm) were used for measurements in the range 650 to 250 nm and 0.5 to 2 mm cells were used in the far UV region. All measurements were obtained with solutions having an absorbance of less than 2 at 280 nm and at 29°C unless otherwise mentioned. Slits were programmed to yield at 10 Å (1 nm) band width at each wavelenth. Mean residue rotations $[\alpha]_{MRW}$ and mean residue ellipticities $[\theta]_{MRW}$ (deg cm²/d mole) were calculated by the standard procedure (Adler *et al.*, 1973). A value of 115 for the mean residue weight (MRW) was assumed.

Carbohydrate content: Carbohydrate was estimated by the method of Montgomery (1961) using 2.5% protein solution in buffer A.

Phosphorus content: Phosphorus was estimated by the method of Taussky and Shorr (1953) using a 2.5% protein solution in 1 M NaCl.

Free and total gossypol content was estimated by the method of Pons and Guthrie (1949) and Pons *et al.* (1950) respectively using a 2.5% protein solution in 1 M NaCl.

Determination of subunits and their molecular weight: The number of subunits in the 7S protein and their molecular weight were determined by analytical SDS-polyacrylamide disc gel electrophoresis (Laemmli, 1970). The following proteins were used as molecular weight markers: bovine serum albumin (68,000), egg albumin (43,000), pepsin (35,000), β-lactoglobulin (18,400) and ribonuclease (13,600).Using 10% separating gel and 3% stacking gel, electrophoresis was carried out in 0.025M Tris-glycine buffer of pH 8.3, containing 0.1% SDS for 2 h at 3 mA/tube; 200-300 μg of the protein was loaded. Gels were stained with 0.25% Coomassie brilliant blue and destained in water containing 7.5% acetic acid and 5% methanol.

Results

Homogeneity of the 7S protein

The homogeneity of the 7S protein was determined by gel electrophoresis, gel filtration and ultracentrifugation. The disc gel electrophoresis pattern of the protein at pH 8.3 showed a single sharp band indicating its homogeneity (figure 1). The protein eluted as a single symmetrical peak in gel filtration (figure 2) with an elution volume of 188 ml. This corresponds to peak II in the gel filtration pattern of cottonseed total protein (figure 3).
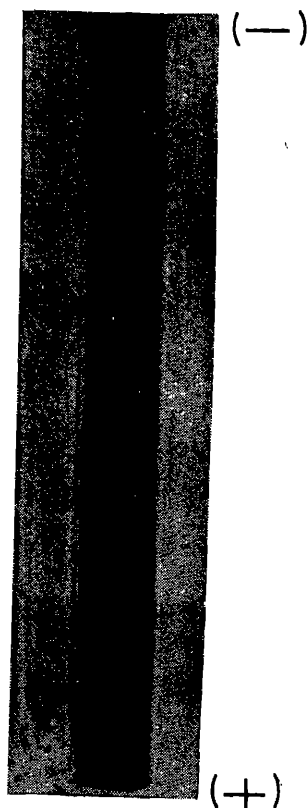
Figure 1. Disc electrophoretic pattern of 7S protein (0.025 M tris-glycine buffer, pH 8.3).
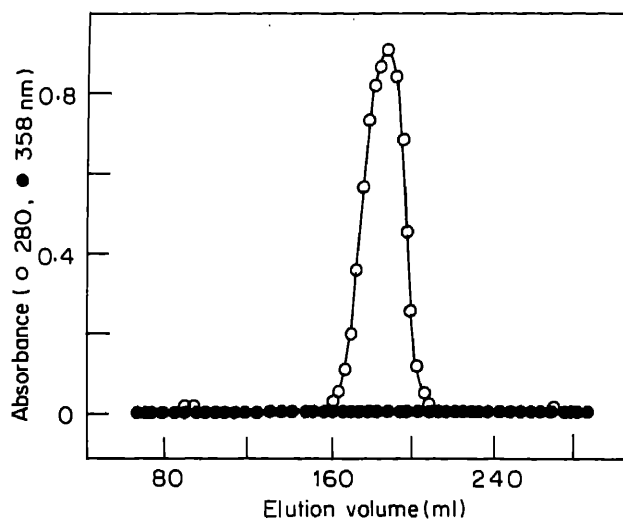
Figure 2. Gel filtration pattern of 7S protein in 1 M NaCl solution on Sepharose 6B-100.
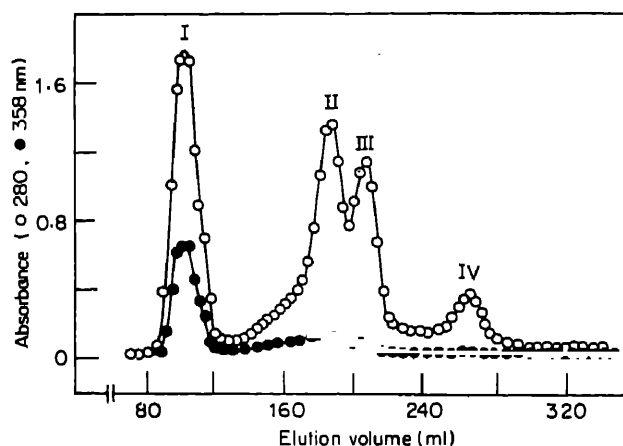
Figure 3. Gel filtration pattern of cottonseed total proteins in 1 M NaCl solution on Sepharose 6B-100.

The sedimentation velocity pattern of the protein in 1 M NaCl consisted of a symmetrical major peak with an $S_{20,w}$ value of 6S and a faster moving component (9.6S) which formed less than 5% of the total (figure 4). Both disc gel electrophoresis and gel filtration did not show the presence of higher molecular weight component in the isolated protein fraction and was homogenous by the
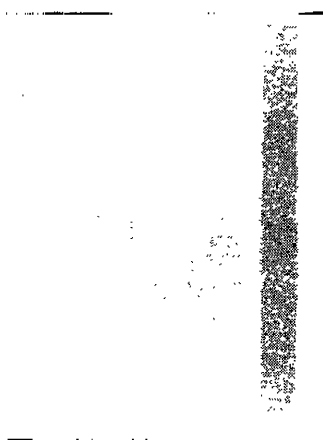
Figure 4. Sedimentation velocity pattern of 7S protein; 1% protein solution in 1 M NaCl. Photograph taken after 60 min at 59,780 rpm; bar angle 65°. Sedimentation proceeds from left to right.

above criteria. Thus by DEAE-cellulose chromatography in buffer A it was possible to isolate the major protein of cottonseed in a homogenous form.

The protein did not contain any phosphorus and was also free from bound and free gossypol. The carbohydrate content of the protein was 1%. Pyrophosphate buffers have been used successfully for minimizing the contamination of nucleic acid and carbohydrate impurities in protein preparations (Eipper, 1972).

The ultraviolet absorption spectrum of the 7S protein is typical of a protein with an absorption maximum at 278 nm and minimum at 250 nm. The ratio of absorbance at 280 to 260 nm was 1.5. This suggested the absence of nucleic acid impurities in the preparation (Layne, 1957) which was also supported by the fact that the protein had no phosphorus. The excitation coefficient of protein at 280 nm, $E_{1\ cm}^{1\%}$, was 6.0.

The protein had fluorescence excitation maximum at 280 nm and emission maximum at 325 nm (figure 5). In proteins containing both tryptophan and tyrosine residues, the fluorescence spectrum of tryptophan alone is observed, irrespective of the wavelength of the exciting light, even in those cases where tyrosine predominates in absorption. The emission maxima of proteins vary between 328 and 342 nm (Teale, 1960). The 7S protein contains 1.28% tryptophan and 2.75% tyrosine (Ibragimov *et al.*, 1974). The results suggest that the fluorescence emission maximum at 325 nm is due to the contribution of tryptophan groups which are in the interior of the protein (Shifrin *et al.*, 1971; Mills and Creamer, 1975).
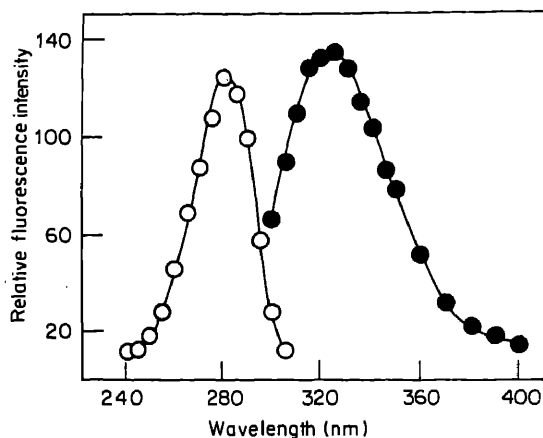
Figure 5. Fluorescence spectrum of 7S protein in buffer A.
—O—excitation spectrum; —●—emission spectrum.

ORD and CD spectra

The CD spectrum of the 7S protein in the region of 300-250 nm (figure 6a) has a positive shoulder at 290 nm and peaks at 285, 280, 265 and 258 nm. The peaks at 258 and 265 nm could be assigned to phenylalanine residues; the peaks at 285 nm
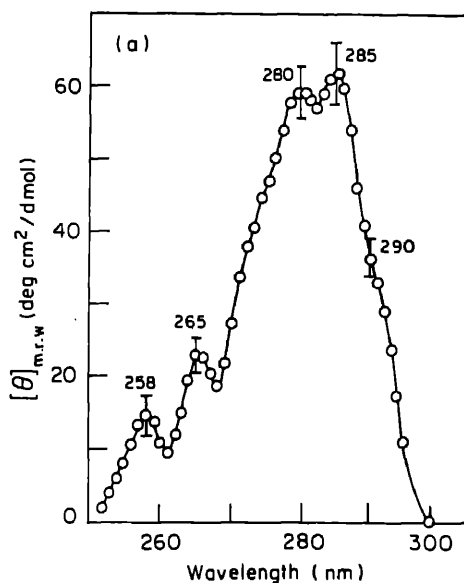
Figure 6a. Near ultraviolet CD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

and 280 nm were possibly due to tyrosine and the shoulder at 290 nm due to tryptophan (Strickland, 1974). Cystine is also known to contribute to the near UV CD spectra of proteins (Strickland, 1974). The 7S protein contains 1.28% tryptophan, 2.75% tyrosine, 6.93% phenylalanine and 1.81% cystine (Ibragimov *et al.*, 1974; Youle and Huang, 1979).

The secondary structure of the 7S protein was determined by measuring its CD spectrum in the region of 260 to 200 nm. The protein exhibited a trough at 208 nm and a shoulder at 225 nm (figure 6b). The helical content of the protein was estimated by the method of Greenfield and Fasman (1969), using the equation

$$\% \ \alpha\text{-Helix} = \frac{-[\theta]208\text{nm} - 4,000}{33,000 - 4,000} \times 100 \qquad (1)$$
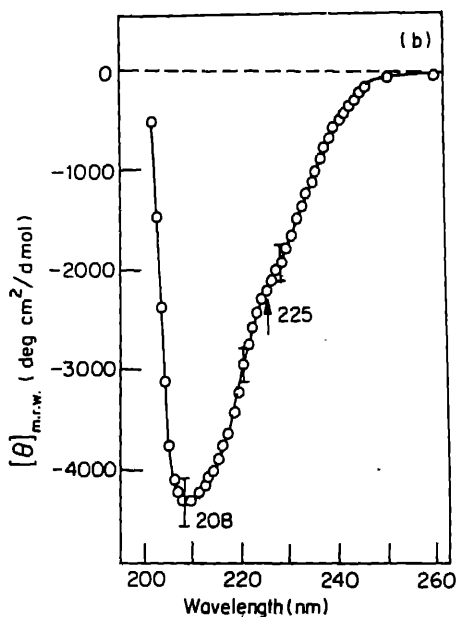
Figure 6b. Far ultraviolet CD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

and by the method of Chen and Yang (1971) using the mean residue ellipticity value at 222 nm, by using the equation

$$[\theta]222\text{nm} = -30,300 \ f_H - 2340 \qquad (2)$$

where f_H = fractional helix content.

Estimation of α-helical content by the two procedures indicated the absence of α-helix in the protein. The proportion of β-structure was estimated by the method of Sarkar and Doty (1966). Taking a mean residue ellipticity value of −23,000 deg

cm²/d-mol at 218 nm for 100% β-structure, the protein was found to contain 16% β-structure. The exact nature of β-structure is not known and the estimate is approximate. Thus, the secondary structure of the 7S protein consists predominantly of β-structure and random coil. These results are comparable to those on the other oilseed proteins (Jacks *et al.*, 1973; Koshiyama and Fukushima, 1973; Jayarama Shetty and Narasinga Rao, 1976; Appu Rao and Narasinga Rao, 1977; Prakash *et al.*, 1980; Gururaj Rao and Narasinga Rao, 1981; Rahma and Narasinga Rao, 1981), which are characterized by low α-helical content and predominant β-structure and random coil. Further studies are in progress to characterise the nature of the β-structure of the protein.

Figure 7 shows the far UV-ORD spectrum of the 7S protein in the region 280-210 nm. The protein exhibited a trough at 233 nm, a shoulder at 221 nm and a cross over point at 215 nm. A similar trough (230-235 nm) has been reported with other oilseed proteins (Fukushima, 1968; Catsimpoolas *et al.*, 1970; Appu Rao and Narasinga Rao, 1976; Appu Rao and Narasinga Rao, 1977; Jayarama Shetty and
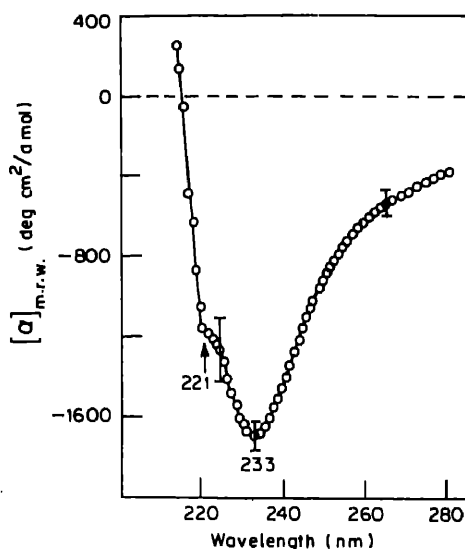
Figure 7. Far ultraviolet ORD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

Narasinga Rao, 1978; Gururaja Rao and Narasinga Rao, 1978; Gururaja Rao and Narasinga Rao, 1981). The trough at 233 nm could be due to either α-helix or anti-parallel β-structure (Fasman and Potter, 1967). The CD measurements suggest that the protein contains very little of α-helix. So the observed trough at 233 nm could possibly be due to antiparallel β-structure. Using a value of −6400 deg cm²/d mol at 230 nm for 100% β-structure (Sarkar and Doty, 1966), the proportion of β-structure in the 7 S protein was estimated as 25%. This could be an overestimate due to contribution of both the antiparallel β-structure and random coil to the rotation at this wavelength.

The optical rotation was also measured in the region 650-330 nm and the data analyzed by the Moffitt-Yang equation (Moffitt and Yang, 1956) to determine the secondary structure.   The equation is

$$[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \tag{3}$$

where $[\alpha]$ is the specific rotation at wavelength λ and λ_0, a_0 and b_0 are constants. Using λ_0=212 nm, $[\alpha]\,[\lambda^2-\lambda_0^2]/\lambda_0^2$ was plotted against $\lambda_0^2/[\lambda^2-\lambda_0^2]$. The slope, b_0, was found to be zero, which indicated the absence of α-helical structure in the protein.

Analytical SDS-polyacrylamide disc gel electrophoresis of 7 S protein in 0.025 M tris-glycine buffer of pH 8.3 containing 0.1% SDS (figure 8) showed that it contained atleast seven non-identical subunits and the molecular weights were 66,000; 57,000; 37,000; 34,000; 21,300; 19,800 and 18,000.
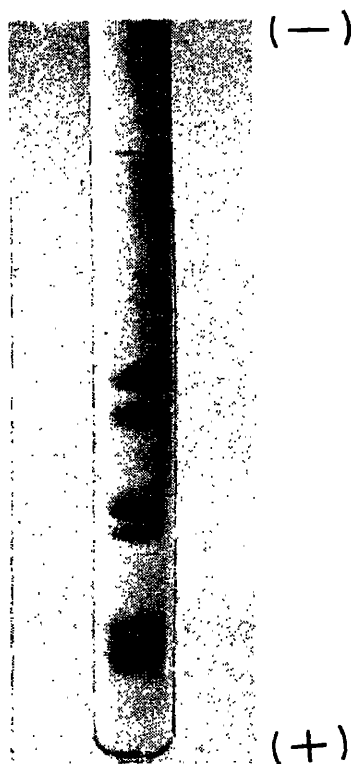
Figure 8. SDS-polyacrylamide disc gel electrophoresis pattern of 7S protein (0.025 M tris-glycine buffer of pH 8.3, containing 0.1% SDS).

Ibragimov *et al.* (1969) and Martinez (1979) have reported the molecular weight of the 7 S protein as 140,000. Since in SDS-PAGE experiments, the bands were not of equal intensity and the stoichiometry of the subunits was not established, no attempt was made to determine the molecular weight from these experiments.

Discussion

The methods reported in the literature for the isolation of the 7 S protein of cotton-seed use (1) the effect of salt and temperature on the solubility of the protein fractions; (2) repeated chromatography on ion-exchange resins or (3) sucrose-density gradient centrifugation. Some of the procedures exposed the protein to extreme changes in pH and temperature. The method described in this report avoids the use of extremes of pH or temperature. Further the various authors do not state if the isolated 7S protein was free from gossypol and nucleic acid impurities. Apart from other effects, bound gossypol can alter the conformation of the protein (Maliwal, Appu Rao and Narasinga Rao, unpublished data). In the present investigation we obtain a 7S protein preparation which is free from bound gossypol (figure 2). This observation is supported by CD spectral evidence also. When gossypol binds to bovine serum albumin it induces extrinsic CD bands at 385-390 nm and 300 nm (Maliwal, Appu Rao and Narasinga Rao, unpublished data). The CD spectrum of the 7S protein in the region 400-300 nm (not shown in Figure 6a) did not indicate peaks at 390 or 300 nm. Thus the method appears to be superior to the earlier methods, especially when the protein is used for conforma-tional studies.

The colouring matter contributed mainly by gossypol and gossypol-like pigments was removed on the DEAE-cellulose column. As mentioned earlier, sodium pyrophosphate buffer seems to have minimised the contamination of nucleic acids and carbohydrate impurities during isolation.

There is some variation in the sedimentation coefficient values reported earlier for the 7 S protein. Species variation, differences in the method of isolation of the protein and the conditions used for ultracentrifugation could have contributed to these variations. The observed $S_{20,w}$ value of the protein in 1 M NaCl at 1% protein concentration was 6 S. Rossi-Fanelli *et al.*, (1964) have reported an $S_{20,w}^{o}$ value of 9.2S in 0.3M NaCl at pH 7.0; Naismith (1956) has reported an $S_{20,w}$ value of 8S in phosphate-NaCl buffer of pH 7.8 and I=0.5; Ibragimov *et al.* (1969) have reported a sedimentation constant of 7 ±0.25 S in 1.25 M NaCl at pH 7.3; and Youle and Huang (1979) have reported, by the density gradient technique, a value of 5 S in 1 M NaCl.

SDS-PAGE experiments showed that the protein consisted of atleast 7 non-identical subunits with molecular weights ranging from 66,000 to 18,000. Further work is in progress to quantitate their proportion and to characterize them.

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Theoretical studies on β-lactam antibiotics VI*: Conformational analysis and structure-activity relationships of penicillin sulfoxides and cephalosporins

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Abstract. Conformational energy calculations were carried out on penicillin α- and β-sulfoxides and Δ^2- and Δ^3- cephalosporins, in order to identify the structural features governing their biological activity.

Results on penicillin β-sulfoxide indicated that in its favoured conformation, the orientation of the aminoacyl group was different from the one required for biological activity. Penicillin α sulfoxide, like penicillin sulfide, favoured two conformations of nearly equal energies, but separated by a much higher energy barrier. The reduced activity of the sulfoxides despite the nonplanarity of their lactam peptide indicated that the orientations of the aminoacyl and carboxyl groups might also govern biological activity.

Δ^3- cephalosporins favoured two conformations of nearly equal energies, whereas Δ^2 cephalosporins favoured only one conformation. The lactam peptide was moderately nonplanar in the former, but nearly planar in the latter. The differences in the preferred orientations of the carboxyl group between penicillins and cephalosporins were correlated with the resistance of cephalosporins to penicillinases.

Keywords. Cephalosporins; penicillin sulfoxides; conformational analysis; structure activity relations.

Introduction

Penicillins, one of the most widely used anti-microbial agents, is known to act by inhibiting the enzyme(s) transpeptidase(s) and/or carboxypeptidase(s), which bring about the cross-linking reaction in peptidoglycan biosynthesis (Blumberg and Strominger, 1974). It has been suggested (Tipper and Strominger, 1965) and subsequently shown theoretically (Virudachalam and Rao, 1977) that penicillin is a structural analog of the natural substrate X-D-Ala-D-Ala.

The widespread use of penicillins has lead to the proliferation of penicillin resistant bacteria: these contain enzymes (penicillinases) which inactivate penicillins by hydrolysing the lactam peptide bond of the drug. Hence, a search has been on for developing drugs active against penicillin resistant bacteria. Cephalosporins, a class of compounds similar to penicillins, have been found to be

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resistant to the action of penicillinases, though their activity is low compared to penicillins. A detailed conformational analysis of cephalosporins, and their comparison with penicillin and its derivatives, is likely to throw light on the stereochemical basis of the resistance of cephalosporins towards penicillinases and their reduced antimicrobial activity.

It has also been observed that minor substitutions in the thiazolidine ring (as in penicillin α- and β- sulfoxides), can drastically reduce the biological activity: the reasons for this, however, are not clearly understood. In order to elucidate the structure-activity relationships in β-lactam antibiotics, conformational analysis of penicillin α- and β-sulfoxides has also been carried out, in addition to Δ^3- and Δ^2- cephalosporins.

Energy calculations

Choice of parameters

The molecule of penicillin sulfoxide is shown in figure 1, and the parameters used to describe it are shown in figure 2. Conformation of the thiazolidine ring is
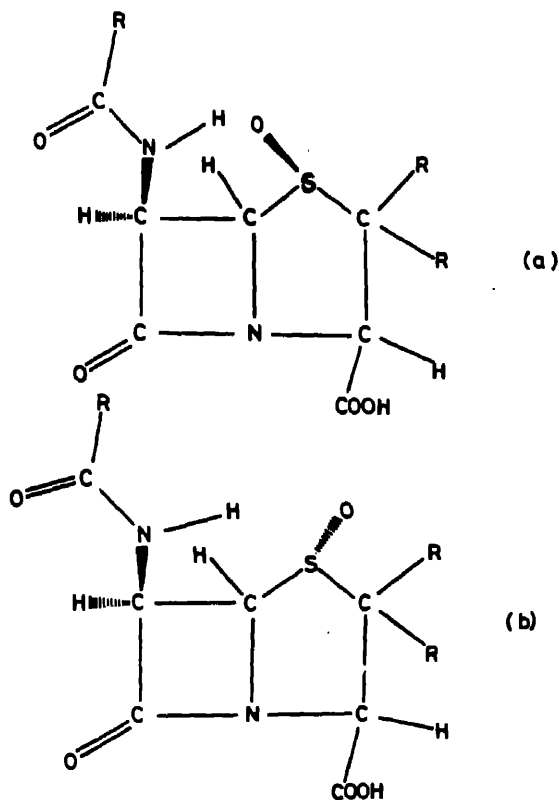
Figure 1. Structure of penicillin sulfoxide.

a . Penicillin β-sulfoxide.
b Penicillin α-sulfoxide. R denotes a methyl group.
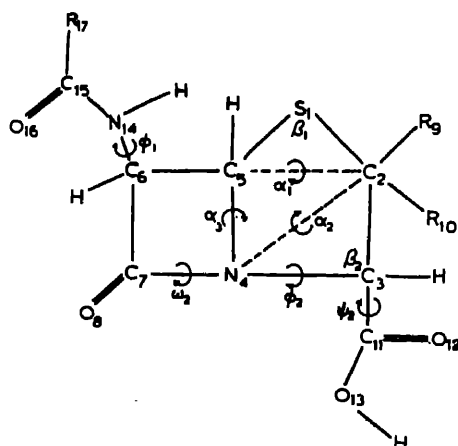
Figure 2. Numbering of atoms and conformational parameters for the thiazolidine ring. R denotes a methyl group.

described in terms of the two dihedral angles a_1 and a_2, denoting rotations about the virtual bonds C-5—C-2, C-2—N-4 respectively. The angle a_3 denotes the relative orientation of the lactam ring (assumed planar) with the C-5-C-2-N-4 plane. The aminoacyl group was fixed using the torsional angle θ_1(C-15-N-14-C-6-C-7). Conformation of the sulfide molecule was thus completely specified by the parameters shown in figure 2. The bond lengths were kept constant throughout the calculations, using crystal structure values.

Figures 3 and 4 depict Δ^3- and Δ^2-cephalosporin respectively. The parameters used to describe the conformation of the six membered dihydrothiazine ring are
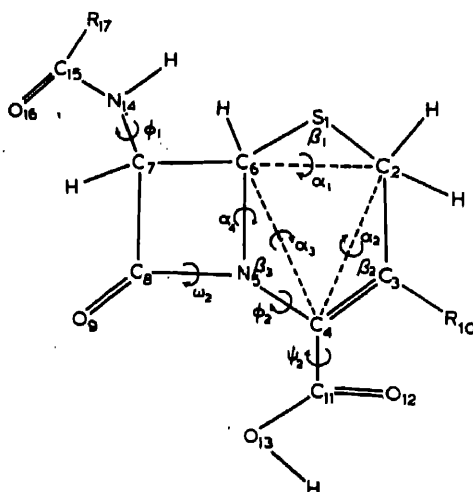
Figure 3. Conformational parameters for Δ^3-cephalosporin. R denotes a methyl group.

similar to those for the sulfoxide and are also shown in figures 3 and 4. Thus, a_1, a_2 and a_3 describe the six membered ring, while a_4 denotes the relative orientations between the lactam ring and the C-4-N-5-C-6 plane. The bond lengths were kept constant at the values obtained from crystal structure. The angle at the aminoacyl end, θ_1 was also kept constant at 160°, as it is energetically favoured, and also because it is unlikely to be affected by changes in the conformation of the dihydro-thiazine ring. ψ_2 was kept constant at 30° as observed in simple peptides (Virudachalam and Rao, 1977).
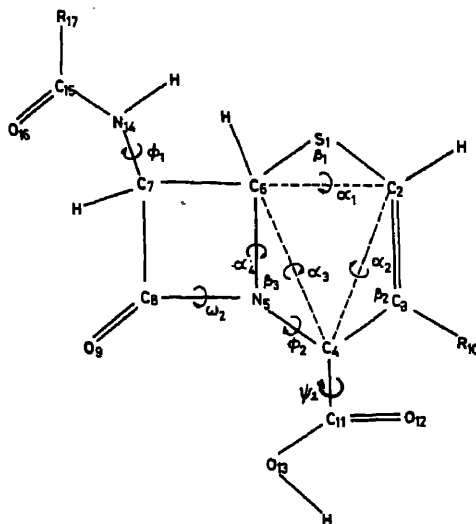
Figure 4. Conformational parameters for Δ^2-cephalosporin. R denotes a methyl group.

Conformational energy calculations

The total conformational energy was computed taking contributions from electro-static and nonbonded interactions, as well as from bond angle and torsional angle distortions. The fractional charges on the atoms were taken to be the sum of the σ-*charges* (obtained by Del Re's (1958) method and π- charges (obtained using Huckel M O theory). Kitaigorodosky's (1961) functions were used to compute the nonbonded interaction energy. In the case of sulfoxides, to estimate the energy of hydrogen bond formation between the sulfoxide oxygen and aminoacyl N-H, the function proposed by Momany *et al.* (1975) was used. The other functions, as well as all the constants used in the present work, have been described earlier (Joshi and Rao, 1979; Joshi, 1980).

It is known from the earlier studies (Joshi *et al.*, 1978) that varying a_1 and a_2 over the range $-70°$ to $70°$ is adequate for sampling all the sterically allowed confor-mations of the thiazolidine ring. Hence, in the present work, a_1 and a_2 were varied over this range at 10° intervals. At every grid point (a_1, a_2), conformational energy was minimised with respect to β_1, β_2, a_3 and θ_1. Isoenergy contours were drawn in the a_1-a_2 plane.

Three variables (a_1, a_2 and a_3) are required to specify the conformation of the six membered dihydrothiazine ring in cephalosporins. However, due to the double bond, for a given (a_1, a_3), the range of a_2 is considerably restricted. Hence, in the present work, a_1 and a_3 were varied over a range $-70°$ to $70°$. At every grid point (a_1, a_3), a_2 was varied over a restricted range, and at each point, the energy was minimised with respect to the bond β_1, β_2, β_3 and with respect to the angle between the rings, a_4. Conformational energy surface of the dihydrothiazine ring was represented by isoenergy contours in the a_1-a_3 plane. A point on such a map corresponds to a conformation whose energy has been minimized with respect to β_1, β_2, β_3, a_4 and a_2. To indicate the small but significant variations in a_2 for a few low energy conformation the values of a_1, a_2, a_3 and the conformational energies were shown in tables 2 and 3 for Δ^3- and Δ^2-cephalosporins respectively.

Results and discussions

The conformational energy map of penicillin a-sulfoxide is shown in figure 5a. There are two minima, the global minimum occurs at (a_1, a_2) = ($40°$, $15°$) and a local minimum at ($-20°$, $-25°$), which is about 0.5 Kcal. mol^{-1} higher in energy than the former. The global minimum corresponds to the C_2 puckered conformation of the thiazolidine ring, and the local minimum to the C_3 puckered conformation. For penicillin a-sulfoxide, no hydrogen bond is possible between the sulfoxide oxygen and aminoacyl N-H.
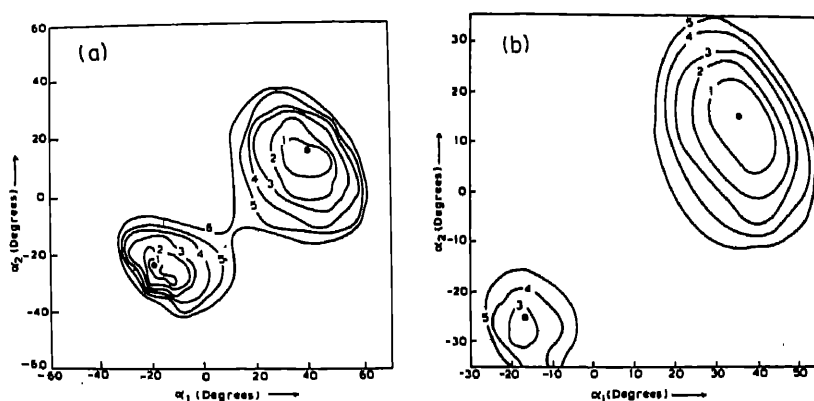
Figure 5. Conformational energy map.
a . Penicillin a sulfoxide.
b. Penicillin β sulfoxide.
Numbers on contours indicate energy in K cal mol^{-1}. Positions of the two minima are marked.

Table 1 shows the angles θ_1, θ_2 and ω_2 for penicillin sulfide, penicillin a-sulfoxide and penicillin β-sulfoxide. It is seen that for penicillin a-sulfoxide also, the lactam peptide is significantly nonplanar ($\omega_2 \sim 132°$) as in penicillin sulfide. Sweet and Dahl (1970) have proposed that the nonplanarity of the lactam peptide bond plays a

Table 1. Calculated dihedral angles of the amino acyl group, carboxyl group and the lactam peptide at the minimum energy conformations for penicillin sulfide and sulfoxides.

Conformation	Favoured orientation of		Non-planarity of the lactam peptide ω_2 (degrees)
	aminoacyl group θ_1 (degrees)	carboxyl group a θ_2 (degrees)	
Penicillin sulfide C$_2$ puckered b	170	111	133
Penicillin sulfide C$_3$ puckered b	170	161	131
Penicillin α-sulfoxide C$_2$ puckered	170	108	131
Penicillin α-sulfoxide C$_3$ puckered	170	157	132
Penicillin β-sulfoxide C$_2$ puckered	−120	110	131
Penicillin β-sulfoxide C$_3$ puckered	−75	157	133

a $\psi_2 = -30°$ (see text.)

b (Joshi *et al.*, 1978).

major role in governing the biological activity of these antibiotics. By this criterion, penicillin α-sulfoxide should be as active as penicillin G or V; on the contrary, the sulfoxides are known to be much less active compared to the sulfides (Gorman and Ryan, 1972).

As can be seen from figure 5a the energy barrier separating the two minima is about six Kcal. mol^{-1}, which is higher by about 2 Kcal. mol^{-1}, than that obtained for penicillin sulfide. As a result, the rate of interconversion between the C$_2$ and C$_3$ puckered forms is about 20 times slower for the sulfoxides compared to the sulfides. This suggests that in solution, as the population of the biologically active C$_3$ puckered conformation decreases due to the interaction with the cross-linking enzymes, the rate at which it is restored due to the conversion from C$_2$ to C$_3$ puckered conformation will be much slower than that for the sulfide. This may account for the reduced activity of penicillin α-sulfoxide.

In penicillin β-sulfoxide the global minimum (figure 5b) occurs at (35°, 15°) as in penicillin α-sulfoxide and in penicillin sulfide, and corresponds to the C$_2$ puckered conformation. There is a possibility of hydrogen bond formation between the sulfoxide oxygen and the aminoacyl N-H groups. This is in agreement with X-ray crystal structure studies (Copper *et al.*, 1969).

The local minimum occurs at $(-15°, -25°)$, and corresponds to the C_3 puckered conformation. In this conformation also, a hydrogen bond between sulfoxide oxygen and the aminoacyl N-H group is possible. However, this conformation has about 2.5 Kcal. mole^{-1} higher energy than the global minimum. The energy barrier separating the two minima is about 7 Kcal. mol^{-1}.

Table 1 shows that the lactam peptide bond is nonplanar ($\omega_2 = 131°$) in both the conformations. Thus, if nonplanarity of the lactam peptide alone is important for biological activity, penicillin sulfide and both the sulfoxides should show more or less the same degree of biological activity. The fact that the activity of β-sulfoxide is considerably lower than that of α-sulfoxide suggests that other conformational features (such as orientations of aminoacyl and carboxyl groups) in the molecule may also have an important role to play in the biological activity.

Since the C_2 puckered conformation of β-sulfoxide is 2.5 Kcal. mole^{-1} lower in energy than the C_3 puckered conformation, in solution the population of the C_3 puckered conformation would be negligible. In fact, from NOE studies (Cooper *et al.*, 1969) it has been shown that penicillin β-sulfoxide exists in solution in the C_2 puckered conformation, which is different from the one required for biological activity. In addition to this, in the minimum energy conformation, the aminoacyl group favours a conformation ($\theta_1 \sim -90°$) different from the biologically active one ($\theta_1 \sim 180°$). This explains the greatly reduced activity of penicillin β-sulfoxide compared to the α-sulfoxide.

Thus, on the basis of a detailed conformational analysis of the thiazolidine ring, the present study consistently explains both, the reduced biological activity of penicillin sulfoxides relative to the sulfides, and also the difference in the activities of the two sulfoxides.

The conformational energy map of Δ^3-cephalosporin is shown in figure 6a. The positions of minimum energy conformations, and the solid state conformation as
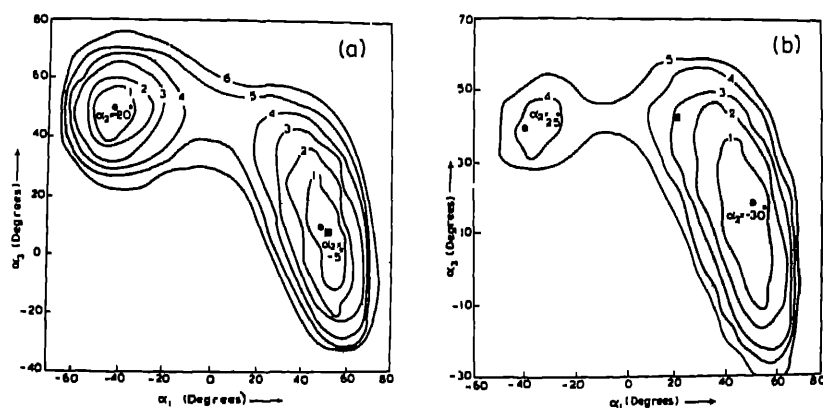
Figure 6. Conformational energy map.

a. Δ^3-cephalosporin and   b. Δ^2-cephalosporin

Numbers on contours indicate energy in Kcal mol^{-1}. Positions of the two minima are marked. The value of α_2 (in degrees) at the minimum energy conformation is also shown in the figure. The solid state conformation is denoted by ■

observed in the crystal structure are also marked in the diagram. The global minimum occurs at (a_1, a_2, a_3) $(-40°, -20°, 50°)$. The local minimum occurs at $(50°, -5°, 10°)$ and is 0.4 Kcal. mole^{-1} higher in energy than the global minimum (table 2). Since this energy difference is small, the dihydrothiazine ring can

Table 2. Conformational angles and energies of some of the low energy conformations of Δ^3 cephalosporin.

No.	a_1 (degrees)	a_2 (degrees)	a_3 (degrees)	E Kcal. mol^{-1}
1	−40	−20	50	0.00 [a]
2	−50	−20	50	0.70
3	−40	−25	60	0.90
4	−30	−20	50	0.90
5	−40	−15	40	1.00
6	50	−5	10	0.40 [b]
7	50	−5	20	0.40
8	50	−5	0	0.70
9	60	−5	0	0.70
10	60	0	−10	0.90
11	60	0	10	1.10

[a] Global minimum.   [b] Local minimum.

assume either of the puckered forms. The solid state conformation (Sweet and Dahl, 1970) corresponds to $(47°, -8°, 8°)$ and lies near the local minimum, indicating that the small energy difference may have been offset by lattice energy. The energy barrier separating the two minima is low (4-5 Kcal. mol$^-$), suggesting that the ring can easily flip over from one conformation to the other. Hence, in solution, both the conformations may exist in considerable proportions.

Recent molecular mechanics calculations of Boyd (1979), have also indicated that Δ^3-cephalosporin can exist in two minimum energy conformations, with the N_5-C_6-S_1-C_2 angle having values 51° and −39°. The present study shows that the values at the local and the global minima are 56° and −21° respectively. However, according to Boyd's study, the latter conformation was about 2-4 Kcal. mol^{-1} higher in energy than the former, whereas the present study indicates that both have nearly equal energies. Since details of the molecular mechanics calculations were not reported, the reasons for the discrepancy between these two results cannot be discussed here.

The present study shows that the lactam peptide is more nonplanar at the global minimum ($\omega_2 \sim 161°$). However, both these are much less nonplanar than that in the penicillins ($\omega_2 \sim 130°$).

The orientations of the carboxyl group at the global and the local minimum are, respectively $\theta_2 \sim 90°$ and $\theta_2 \sim 35°$. Interestingly, these are quite different from those observed for penicillins in either of the conformations ($\theta_2 \sim 160°$ and 110° for C_3 and C_2 puckered conformations respectively).

Conformational energy map of Δ^2-cephalosporin is shown in figure 6b. T position of the energy minima and the solid state conformation are marked in tl diagram. Table 3 shows a_1, a_2 and a_3 for some of the low energy conformations.

Table 3. Conformational angles and energies for some of the low energy conformations of 'Δ^2-cephalosporin.

No.	a_1 (degrees)	a_2 (degrees)	a_3 (degrees)	E (Kcal. mol^{-1})
1	50	−30	10	0.00 [a]
2	50	−30	20	0.00
3	60	−35	10	0.40
4	60	−35	0	0.40
5	50	−30	30	0.40
6	40	−25	30	0.50
7	40	−25	20	0.70
8	60	−35	20	0.90
9	−40	25	40	3.10 [b]
10	−30	20	40	3.40
11	−30	20	50	3.80

[a] Global minimum. [b] Local minimum.

The dihydrothiazine ring of Δ^2-cephalosporin shows two minimum energy co formations. As seen from table 3, the global minimum occurs at ($50°$, $-30°$, $10°$ The local minimum occurs at ($-40°$, $25°$, $40°$) and has about 3 Kcal. mol^{-1} high energy than the global minimum, indicating that the former would be favoure both in solid state and in solution. The conformation observed in the solid sta (Sweet and Dahl, 1970) is similar to the one at the global minimum, but has about 3 Kcal. mol^{-1} higher energy. The value of θ_2 at the global minimum is ~$90°$, an at the local minimum is ~$150°$. At the global minimum, ω_2~$170°$, suggestir that the lactam peptide is less nonplanar, compared to the penicillins. At the loc minimum, ω_2~$143°$, indicating considerable nonplanarity. However, due to i higher energy, this conformation is unlikely to be observed either in the solid sta or in solution.

As mentioned earlier for the Δ^3-cephalosporins, in both the puckered conform tions of the dihydrothiazine ring, the lactam, peptide bond will assume appreciab nonplanarity. On the other hand, in the preferred conformation of Δ^2-cephal sporin, the lactam peptide bond is less nonplanar. This can account for tl observed difference in the biological activities of these compounds.

For Δ^3-cephalosporins, the aminoacyl group assumes approximately the san orientation as in penicillins, but the carboxyl group orientation is different. fact, the values of θ_2 for Δ^3-cephalosporins (~$35°$ and ~$90°$) are very mu different from the one observed in the biologically active conformation penicillin (~$160°$). Since Δ^3-cephalosporin is active (though less compared

penicillin), the differences at the carboxyl group end suggest that the mode o. binding of this drug with the cross linking enzymes may differ slightly from that oi penicillins. Such differences in the mode of binding of molecules which differ in the orientations of some of the groups are not unusual, e.g., binding of α and β anomers of N-acetyl glucosamine to lysozyme (Beddel *et al.*, 1970). However, it is not clear which of the conformations of the dihydrothiazine ring is associated with biological activity.

Our earlier studies (Joshi *et al.*, 1978) on specificity of penicillinases have indicated that for binding to penicillinases, the orientation of the carboxyl group (θ_2) should be 150°; as in penicilin (C_3 puckered) or clavulanic acid. However, in neither Δ^3-nor Δ^2 cephalosporins, the energetically favoured conformations have carboxyl group orientations near this value. These differences in the orientations of the −COOH group perhaps account for the resistance of cephalosporins to penicillinases.

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Mechanism of action of carboxin and the development of resistance in yeast

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Abstract. Carboxin prevents the growth of yeast by inhibiting protein synthesis; the resumption of growth in the presence of this chemical appears to be primarily due to a cellular alteration affecting carboxin entry into the cells.

Keywords. Carboxin; macromolecular synthesis; resistance development.

Introduction

Carboxin (2,3,dihydro-5-carboxanilido-6-methyl 1,4,oxathiin, vitavax) is one of the several systemic fungistatic chemicals used in agriculture to control pathogenic fungi. In recent years, there are reports of resistance development to this chemical (Ogawa *et al.*, 1977) and the mechanism of this resistance has been attributed to the alteration of the target site. For example, in *Ustilago* sp. this chemical is reported to inhibit growth by affecting mitochondrial succinic dehydrogenase and in resistant strains the target site has been found to be altered (Gunatilleke *et al.*, 1976). The use of mycelial fungi which are obligate aerobes, to understand the mechanism of action of fungistatic chemicals and resistance development has certain disadvantages, whereas the ascomycetous yeast *Saccharomyces cerevisiae* provides an ideal test system. The latter is sensitive to most fungistatic chemicals, is a facultative anaerobe and is also amenable to both genetic and biochemical analysis. Using this organism, we have tried to understand the mechanism of action of carboxin and the development of resistance to carboxin. In this paper, we report that in yeast the target site is not the respiratory system and that the development of resistance involves increased entry of the chemical into the cells.

Materials and methods

The haploid yeast strain *S. cerevisiae*, 2180-1B ($a \rho^+$) was obtained from the yeast stock culture centre, University of California, Berkeley, California, USA. Strain 9B (a, ρ^-), a respiratory deficient strain lacking mitochondrial protein synthesis

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was isolated in this laboratory (Tauro, unpublished). These cultures were maintained on yeast extract—agar slants (yeast extract, 0.5%; peptone 1%; dextrose 2% and agar 2%) by regular transfers. *Aspergillus niger* 3/1 was from the culture collection of this department and was maintained on potato dextrose agar slants. Carboxin (97% W.P.) was from M/s Uniroyal Chemical Co., Connecticut, USA. Stock solutions of this chemical were prepared in acetone and diluted as and when required. [^{14}C]-L-thereonine (sp. act 80 mCi/mmol) and [^{14}C]-uracil (sp. act. 46.7 mCi/mmol) were from Bhabha Atomic Research Centre, Bombay. For growth measurement, cells from a 12 h old slant culture were transferred to 50 ml of synthetic medium (Rose and Nickerson, 1956) in 250 ml conical flasks and incubated at 30°C on a rotary shaker (230 rpm). At intervals, 5 ml samples were withdrawn and the growth was measured by determining the absorbance at 620 nm using a Bausch and Lomb Spectrophotometer.

For the measurement of DNA synthesis, a 12 h old culture was diluted to 0.2 absorbance units in 50 ml of synthetic medium in multiple flaks with or without carboxin (200 µg/ml) and incubated at 30°C on a rotary shaker. At intervals of 30 min duplicate flasks were withdrawn and the cells were collected by centrifugation at 1500 g for 10 min at 4°C. The pellet was washed twice with ice cold water and the DNA extracted by the method of Clemons and Sisler (1971). The DNA content of the extract was determined colorimetrically using diphenyl amine reagent (Burton, 1956). For the measurement of RNA and protein synthesis, a 12 h old culture was diluted to 0.2 absorbance with fresh medium and 20 ml of this was treated with either 20 µCi of [^{14}C]-uracil or 20 µCi of [^{14}L]-threonine (for RNA and protein synthesis respectively) in the presence or absence of carboxin (200 µg/ml) and incubated at 30°C on a rotary shaker. Samples (1 ml) were withdrawn at intervals of 10 min into tubes containing 1 ml of 10% cold tricholoroacetic acid and incubated at 4°C for 6 h. The tubes were then centrifuged at 1500 g for 10 min at 4°C and the pellet was washed thrice with a total of 25 ml of 5% cold trichloroacetic acid containing either excess of cold uracil or L-threonine. The pellets were finally suspended in 1 ml of 10% trichloroacetic acid and transferred to scintillation vials containing 10 ml of Bray's scintillation fluid (Hash, 1972). The radioactivity of the sample was determined using an automatic Beckman Liquid Scintillation counter.

Residual carboxin in the growth medium was assayed colorimetrically using the method of Lane (1970) and by bioassay using *A. niger* 3/1. For bioassay, spores were inoculated into cell-free spent medium after removal of yeast cells and determining growth by visible observations. The ultraviolet and infrared spectra of the residual chemical in the growth medium were determined by extracting with chloroform and using a Beckman automatic recording spectrophotometer (Model 25) or an infrared spectrophotometer respectively.

Results

Effect of carboxin on yeast growth

Figure 1 shows the growth of a respiratory component (ρ^+) and a respiratory deficient (ρ^-) strain of *S. cerevisiae* in the presence or the absence of carboxin.
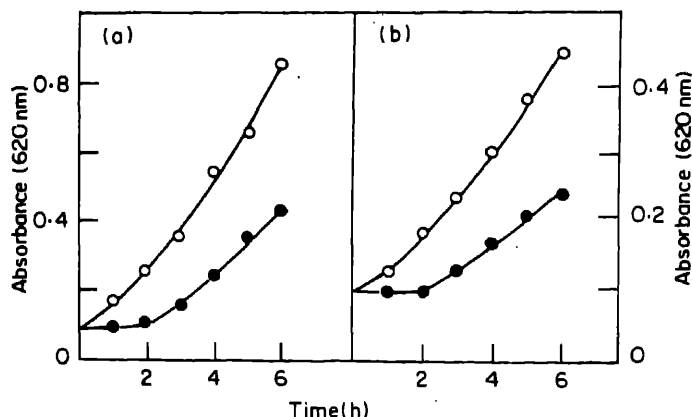
Figure 1. Effect of carboxin on the growth of *S. cerevisiae*. a. *S. cerevisiae* 2180-1B ρ+; b. *S. cerevisiae* 9 Bρ⁻. Control (O); +carboxin (200 µg/ml) (●).

At a concentration of 200 µg/ml carboxin (maximum water solubility level) growth was inhibited in both strains for about 120 min, after which it resumed at a slower rate than in control. The pattern of inhibition was similar both in the respiratory deficient and competent cells suggesting that the respiratory site might not be the target for carboxin in yeast. Therefore in subsequent studies, only strain 9 Bρ⁻ was used.

Effect of carboxin concentration on the duration of growth inhibition

To determine if the duration of inhibition was dependent on the concentration of the chemical, cells were inoculated into media containing 0, 50, 100 and 200 µg carboxin/ml and incubated as before (figure 2). It was found that the duration of
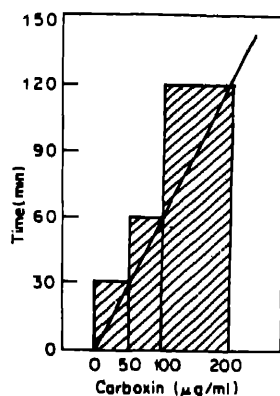
Figure 2. Effect of carboxin concentrations on the period of growth inhibition.

growth inhibition was dependent on the concentration of the chemical in the medium suggesting that the transport of this chemical into the cells occurred perhaps through a passive diffusion process which was concentration dependent.

In later experiments, unless otherwise stated, a concentration of 200 µg of carboxin/ml was used.

Effect of carboxin on macromolecular synthesis

To determine the mechanism of growth inhibition and the exact site of action, the synthesis of DNA, RNA and proteins in the presence and absence of carboxin was determined (figure 3). During the first 60-120 min of exposure to carboxin, DNA
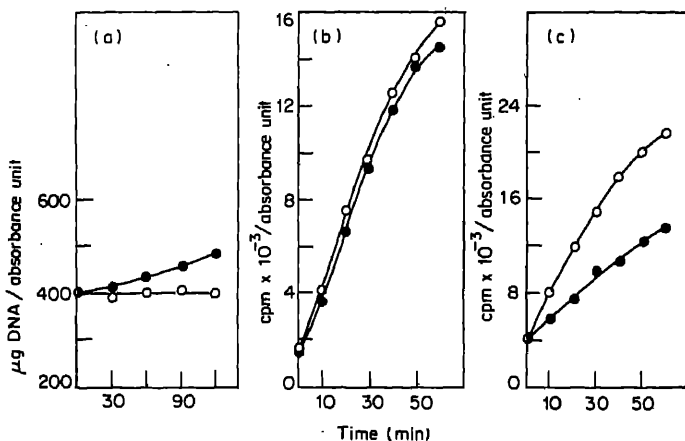
Figure 3. Effect of carboxin on macromolecular synthesis in *S. cerevisiae* 9 B ρ⁻; a. DNA synthesis; b. RNA synthesis; c. Protein synthesis. Control (O); +carboxin (200 µg/ml) (●).

synthesis continued without an increase in absorbance (growth); RNA synthesis was normal while protein synthesis was reduced by about 40-50%. It therefore appeared that the site of action of carboxin might be protein synthesis and the inhibition of growth could have been mainly due to the temporary inhibition of protein synthesis.

Mechanism of resumption of growth

In the presence of 200 µg carboxin/ml growth was inhibited for about 120 min after which it was resumed at a slower rate. The resumption of growth in the presence of carboxin could occur (i) as a result of autodegradation or biodegradation of the chemical or (ii) structural alteration leading to the formation of a nontoxic compound or (iii) due to the development of cellular impermeability (Dekker, 1977). To determine as to which of the above mechanisms was responsible to allow growth in the presence of carboxin, a variety of tests were conducted.

To test for autodegradation, uninoculated flasks containing the chemical were incubated for varying periods of time beyond 2 h and later inoculated with yeast culture and the pattern of growth determined. It was found that the growth was similar to that reported in figure 1 indicating that the chemical does not undergo autodegradation (data not given).

To test for biodegradation or structural alterations leading to detoxification, the culture was centrifuged after the resumption of growth and the clear supernatant was used for determining the level of carboxin colorimetrically or by bioassay. It was found that about 95.5% of the chemical was still present in the growth medium even after 4 h of growth. Further, the ultraviolet and infrared spectra of the chloroform extracted chemical were identical with that of the authentic sample. Also, inoculation of the spent medium with a fresh yeast culture after 3 h of growth showed a pattern of growth similar to that in figure 1 (data not presented). All these results allow us to conclude that the chemical does not undergo any change but that the resumption of growth apparently is due to cellular alteration leading to a decreased permeability to this chemical which allows resumption of protein synthesis.

Specificity of resistance

To determine if the resistance developed against carboxin is specific or nonspecific, cells grown in medium containing carboxin for 4 h were centrifuged and transferred to a medium containing oxycarboxin, a close analogue of carboxin (figure 4). It was seen that cells exposed previously to carboxin were unable to resume growth immediately (figure 5) suggesting that the resistance was specific only to carboxin.

(a)   (b)
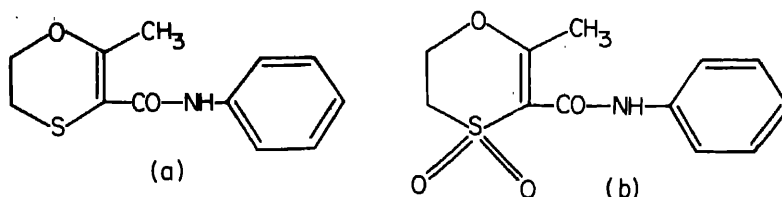
Figure 4. Structure of carboxin and oxycarboxin; a. Carboxin (vitavax): 2,3, dihydro-5-carboxanilido-6-methyl 1,4 oxathiin. b. Oxycarboxin (plant vax): 2,3-dihydro-5-carboxanilido-6-methyl 1,4 oxathiin 4,4-dioxide.
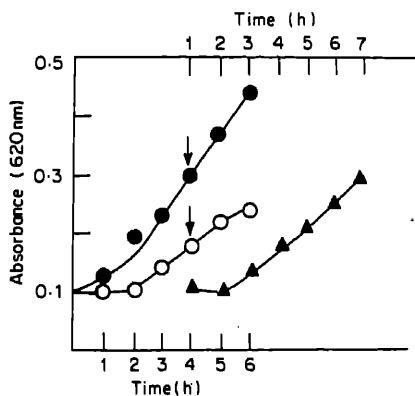
Figure 5. Test for cross resistance against oxycarboxin. Control (O); +carboxin (200 µg/ml) (●); cells transferred from either control or plus carboxin to fresh medium containing oxycarboxin (the arrows indicate the time of transfer), (▲).

Induction of resistance

If the resumption of growth in the presence of carboxin is due to cellular alteration induced by carboxin, then cells exposed to carboxin for various time intervals and retransferred to fresh carboxin medium should show a proportionate reduction in the period of the growth inhibition. To test this, cells from carboxin medium were centrifuged after 1 and 2 h of exposure and resuspended in fresh carboxin medium (figure 6). As expected, cells exposed to carboxin previously showed a
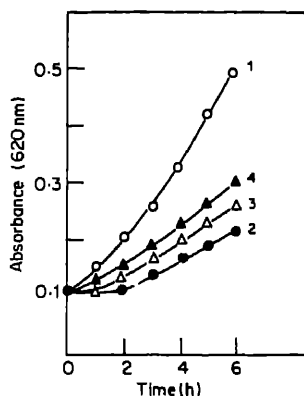
Figure 6. Induction of resistance to carboxin. Control (O); +carboxin (●); cells exposed to carboxin for 1 h and transferred to fresh carboxin medium (△); similar to 3 but cells exposed to carboxin for 2 h (▲). The concentration of carboxin was 200 μg/ml.

proportionate decrease in the duration of inhibition before growth resumed, indicating that the mechanism which allows growth is induced within the first 120 min of exposure to the chemical.

To determine the period for which the induced cells are resistant to carboxin, the cells were grown in carboxin medium for 12 h and then transferred to carboxin free medium. At various intervals the samples .were withdrawn, centrifuged and retransferred to carboxin medium. As a control, the carboxin grown cells were directly transferred to fresh carboxin medium without exposing to carboxin free medium (figure 7). It was found that the ability to grow in the presence of carboxin
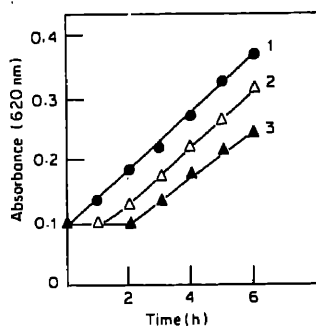
Figure 7. Test for the nature of induced resistance. Carboxin grown cells were retransferred to fresh carboxin medium (●); carboxin grown cells were exposed to carboxin free medium for 30 min and retransferred to fresh carboxin medium (△); similar to 2 but cells were exposed to carboxin free medium for 1 h (▲). The concentration of carboxin was 200 μg/ml.

was lost within 60 min of growth in its absence suggesting that the cellular alteration is conditional and transient.

Discussion

Our studies using respiratory deficient yeast mutant demonstrate that carboxin inhibits growth in ascomycetous yeast primarily by affecting protein synthesis. Alternate mechanisms in other mycelial fungi cannot be completely ruled out.

The resumption of growth in the presence of carboxin was apparently due to a specific alteration that affected the cell permeability to carboxin. Estimation of residual carboxin in growth medium indicated that more than 95% of the chemical was not metabolised. The rate of transport was altered after about 2-3 h of yeast growth in the carboxin medium (Tauro, unpublished data). Examples of such cellular alterations induced by chemicals were not reported in fungi. In *Staphylococcus aureus,* growth in the presence of tetracycline was reported to occur by a similar mechanism involving the synthesis of specific proteins (Levy *et al.,* 1978). Such mechanisms may be quite common in pathogenic fungi and may be induced by both fungistatic and fungicidal chemicals. We have preliminary evidence to show that such cellular alterations leading to decreased permeability to the chemical occur at the level of the cytoplasmic membrane and such mechanisms are under genetic control.

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Induction of riboflavin-carrier protein in the immature male rat by estrogen: kinetic and hormonal specificity

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Abstract. The kinetics of estrogen-induced accumulation of riboflavin-carrier protein in the plasma was investigated in immature male rats using a specific and sensitive homologous radio-immunoassay procedure developed for this purpose. Following a single injection of the steroid hormone, plasma riboflavin-carrier protein levels increased markedly after an initial lag period of approximately 24 h, reaching peak levels around 96 h and declining thereafter. A 1.5 fold amplification of the inductive response was evident on secondary stimulation with the hormone. The magnitude of the response was dependent on hormonal dose, whereas the initial lag phase and the time of peak riboflavin-carrier protein induction were unaltered within the range of the steroid doses (0.1-10 mg/ kg body wt.) tested. Simultaneous administration of progesterone did not affect either the kinetics or the maximum level of the protein induced. The hormonal specificity of this induction was further adduced by the effect of administration of antiestrogens viz., En and Zu chlomiphene citrates, which effectively curtailed hormonal induction of the protein. That the induction involved *de novo*-protein synthesis was evident from the complete inhibition obtained upon administration of cycloheximide. Passive immunoneutralization of endogenous riboflavin-carrier protein with antiserum to the homologous protein terminated pregnancy in rats confirming the earlier results with antiserum to chicken riboflavin-carrier protein.

Keywords. Riboflavin carrier protein; radioimmunoassay; estrogen induction; specificity; kinetics;amplification; antiestrogens; secondary stimulation; immunoneutralization; pregnancy termination.

Introduction

In the chicken (and presumably in other oviparous species also), adequate deposition in the egg of water-soluble vitamins like riboflavin (Rhodes *et al.*, 1959; Murty and Adiga, 1977), thiamin (Muniyappa and Adiga, 1981), biotin (Eakin *et al.,* 1940; White *et al.*, 1976; Murthy and Adiga, 1980) etc. for utilisation by and proper development of the prospective embryo, is accomplished by the obligatory partici-pation of the high-affinity carrier proteins specific to each of these vitamins. Earlier, we showed that all these egg proteins are inducible specifically by estrogen (Murty and Adiga, 1978; Muniyappa and Adiga, 1980a; Ramana Murty and Adiga, unpublished) either in the adult male or immature animals of either sex.

Abbreviations used:RCP,riboflavin-carrier protein; TCP, thiamine carrier protein; BCP, biotin carrier protein.

Notwithstanding the marked difference in the patterns of embryonic development in the oviparous species *vis-a-vis* the mammals, we could demonstrate recently that in the pregnant rat also, a similar gestation-specific, high-affinity carrier-protein with immunological cross-reactivity to the purified chicken riboflavin-carrier protein (RCP), mediates the transplacental transport and foetal accumulation of riboflavin (Muniyappa and Adiga, 1980b; Murty and Adiga, 1981). The rodent RCP was purified to homogeneity (Muniyappa and Adiga, 1980c) and its functional importance in foetal development demonstrated. For example, it was shown that immunoneutralisation of this maternal protein in the pregnant rat by specific antiserum to chicken RCP precipitates acute flavin deficiency in the developing conceptus with a resultant marked foetal wastage followed by termination of pregnancy (Murty and Adiga, 1981). During attempts to understand the endocrine basis of the elaboration of this protein in the rat, we found using hetero-logous radioimmunoassay, that RCP levels in the cycling female rats were modulated in synchrony with the changing estrogen levels and that the steroid hormone could induce this protein *de novo* in ovarectomised female rats (Muniyappa and Adiga, 1980b). In the present paper, we report a homologous radioimmunoassay for estimating RCP and provide information on the kinetics of its induction by estradiol-17β in the immature male rat in response to both primary and secondary stimulation as well as the influence of specific hormonal antagonists and other factors thereon. Availability for the first time of the potent antiserum to the rodent protein prompted a reinvestigation of the effect of passive immuno-neutralisation of RCP on the course of pregnancy in this mammal.

Materials and methods

Thirty day old male rats (Wistar) of our Institute Colony were fed a pelleted diet (Hindustan Lever Products, Bombay) and water *ad libitum* and exposed to a 14h:10h light-dark schedule. The purification of RCP from pregnant rat serum was accomplished according to the published procedure (Muniyappa and Adiga, 1980c). The antiserum against the purified rat RCP was raised in rabbits by subcutaneous injections of the protein (100 μg) emulsified with Freund's complete adjuvant, once in a week, for 3 weeks followed by a booster dose of the protein (200 μg) in saline. A week after the booster, blood was collected through the ear vein, and the serum prepared and kept frozen at $-20°C$. Na ^{125}I for iodination of the protein, was purchased from the Radiochemical Centre, Amersham, Bucks, U.K. The sources of other chemicals and reagents were the same as described earlier (Muniyappa & Adiga, 1980). The female rats used in passive immunoneutralisa-tion experiments were 80-100 day old, and day 1 of pregnancy was the day on which the sperms were detected in the vaginal smear. Antiserum (0.5 ml) was injected intraperitoneally on the 11th day of pregnancy, after inactivating the complement in the serum by warming in a water bath at $52°C$ for 5 minutes. Laprotomy was performed on day 8 to count the number of implantation sites on their uterine horns. Hormones and antiestrogens in propane-1,2-diol were injected intra-muscularly, whereas cycloheximide in 0.15M NaCl was administered intraperi-tonially.

Homologous radioimmunoassay for riboflavin carrier protein

The iodination of RCP was carried out at 4°C by the procedure of Greenwood *et al.* (1963). Briefly, 2.5 µg of the protein in 25 µl of 100 mM-sodium phosphate buffer, pH 7.2 was treated for 45 s with 0.25 mCi of carrier-free Na ^{125}I in the presence of 25 µl of chloramine T (1 mg/ml) and the iodination was terminated with the addition of 25 µl of sodium metabisulphite (10 mg/ml). The iodinated protein was separated from the free iodine using a column (0.6 cm × 20 cm) of Sephadex G-75 pre-equlibrated with 0.15 M NaCl in 10 mM-sodium phosphate buffer, pH 7.5 containing 0.5% (w/v) gelatin. The presence of gelatin ensured the complete recovery of the protein from the column. Small portions (25 µl) from each fraction (1 ml) were counted for radioactivity in a gamma counter (Packard Autogamma, Model 2002). The iodinated protein was stored frozen in small batches in the presence of 2% (w/v) bovine serum albumin until use.

Immunoassay procedure

An antiserum dilution capable of binding 25-40% of the labelled protein was selected. The assay was performed in glass tubes (Moudgal and Madhwaraj, 1974) at 37°C in a water bath. Rat RCP (1-200 ng) standards duplicates, and 0.05-0.1 ml portions of unknown samples were incubated with antiserum (0.1 ml) along with non-immunised rabbit serum as controls, for 10-12 h. Labelled protein (*Ca* 50,000 cpm) was added to all the tubes and the incubation was continued for another 12 h and the antigen-antibody complex was precipitated by the double-antibody method (Murthy and Adiga, 1978) in the presence of a carrier viz. 0.1 ml of 1:50 diluted normal rabbit serum. The precipitate was sedimented by centrifugation at 2000 *g* for 20 min and the radio activity measured. The amounts of RCP in the unknown samples were calculated by extrapolation from the standard curve (figure 1) that was constructed using various known amounts of unlabelled protein
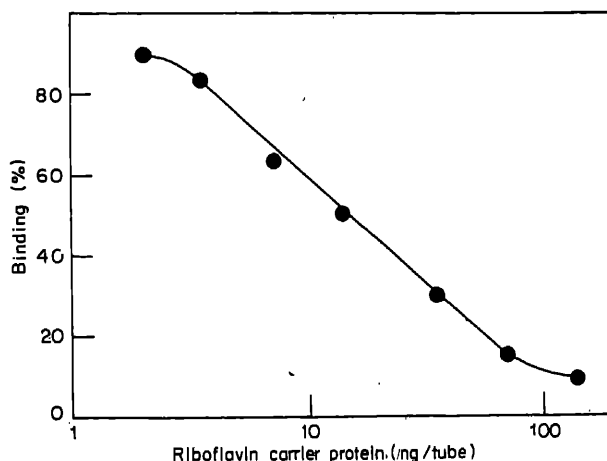
Figure 1. Standard curve for the radioimmunoassay for rat riboflavin carrier protein. The dilution of the antiserum used was 1:5000. For other details see the text.

to inhibit the binding of the labelled protein. The total bound radioactivity in the protein refers to the amount of specifically-bound radioactivity in the absence of unlabelled protein. The non-specific binding (to non-immunised rabbit serum) was 5-10% of the total input radioactivity and this was always subtracted.

Inter assay and intra assay variations:

Aliquot from a single pooled serum sample was analysed 4-8 times for intra-assay and 4 times for inter-assay variation and the data were analysed statistically (Mulholland & Jones, 1968). Coefficients of intra and inter-assay variations were 2-6% and 3-5% respectively and the assay method had a sensitivity of 3.5 ng/tube. The homologous radio-immunoassay reported here had approximately 2 fold higher sensitivity compared to the earlier heterologous radioimmunoassay (Muniyappa and Adiga, 1980b).

Results

The preliminary evidence (Muniyappa & Adiga, 1980b) that the rat RCP, like its avian counterpart, is inducible in the mammalian liver by estrogen was based on the measurement of circulating levels of this protein in the steroid-treated overectomized female rats using a heterologous radioimmunoassay which reflected only the relative concentrations of the protein in the plasma. To assess the absolute content of this vitamin-carrier following estrogenization, a sensitive radioimmunoassay was evolved after the purified rat RCP and its specific antiserum became available. Having accomplished this, a detailed investigation of the time course of accumulation of this protein in the plasma after hormonal dosing was undertaken. The time-course of accumulation and disappearance of RCP during both primary and secondary stimulations with the steroid (10 mg/kg body weight) in 30-day old male rats is depicted in figure 2. The plasma levels of the vitamin-carrier measured prior to hormone treatments represented the respective base-line values which were low (30-50 ng/ml). The increments, if any, in these
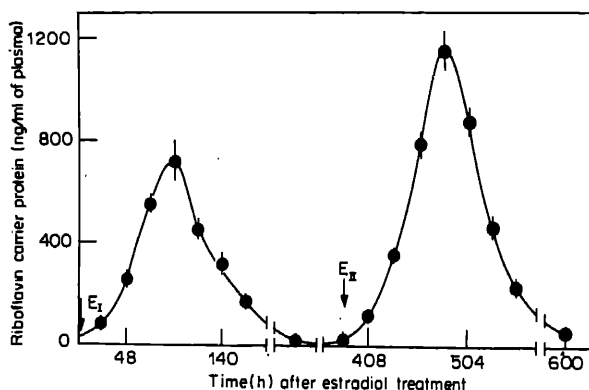
Figure 2. Time course of accumulation of riboflavin carrier protein in the plasma after sequential injections (E_I, E_{II}) of oestradiol-17β in propane-1,2-diol (10 mg/kg body wt). The values represent means and vertical bars represent S.D. (n=5).

levels were not significant for nearly 24h after estrogen treatment. Beyond this initial lag period, the RCP concentrations rose gradually with time reaching a peak value of approximately 750 ng/ml at around 96h in conformity with the earlier data (Muniyappa and Adiga, 1980b). This was followed by a steady decline and by day 10, the levels returned to the preinjection base-line values.

Sixteen days after withdrawal of the steroid, when the same animals were again challenged with a second dose of estradiol-17β (10 mg/kg body weight) the circulating concentration of RCP exhibited a similar kinetic pattern except that the response was considerably magnified; in fact, a typical 'memory effect' observed earlier during the elaboration of vitellogenin (Gruber *et al.*, 1976), RCP (Murthy and Adiga, 1978), thiamine-carrier protein (Muniyappa and Adiga, 1980a) and biotin-carrier protein (Ramana Murty and Adiga, unpublished observations) in estrogen treated chicken liver was clearly evident. During this secondary stimulation, the amplification of the estrogenic response in terms of RCP induction was more than 1.5 fold. Though the peak plasma concentration of RCP was enhanced by a similar order of magnitude during the secondary stimulation, the time-course of accumulation and disappearance of the protein in circulation exhibited qualitatively the same pattern following the two hormonal stimuli. However, a comparison of the slopes of the respective ascending phases of RCP accumulation clearly shows that the rate of induction, especially during early phases, is significantly enhanced after secondary stimulation *vis-a-vis* primary stimulation.

Hormonal dose-dependency and time-course of RCP elaboration during primary stimulation

Varying doses of estradiol-17β were administered to young male rats during the primary stimulation to quantify the corresponding changes in the rates and the magnitudes of plasma accumulation and disappearance of RCP. It is evident from figure 3 that as low a dose as 100 µg/kg body weight (i.e., less than 10 µg/animal) of the steroid could induce a measurable response which progressively increased with the hormonal dose. However, the time taken to reach the maximal protein levels following hormonal dosing remained the same within the range of the steroid dosage tested (0.1-10 mg/kg body weight). But the rates of accumulation and disappearance from circulation as well as the peak levels reached exhibited a dose-dependent phenomenon.

Influence of cycloheximide

One possibility that the rapid accumulation of RCP in the plasma after hormone administration, merely reflects an interference with the altered metabolic clearance rate and not necessarily *de novo* synthesis was tested by administration of cyclo-heximide, a powerful inhibitor of protein synthesis. When this drug was injected intraperitoneally (0.8 mg/kg body weight) 30 minutes prior to steroid administration a drastic decrease in RCP levels was observed at 72h, i.e., at a time when many fold increase in RCP concentration was otherwise encountered (table 1).
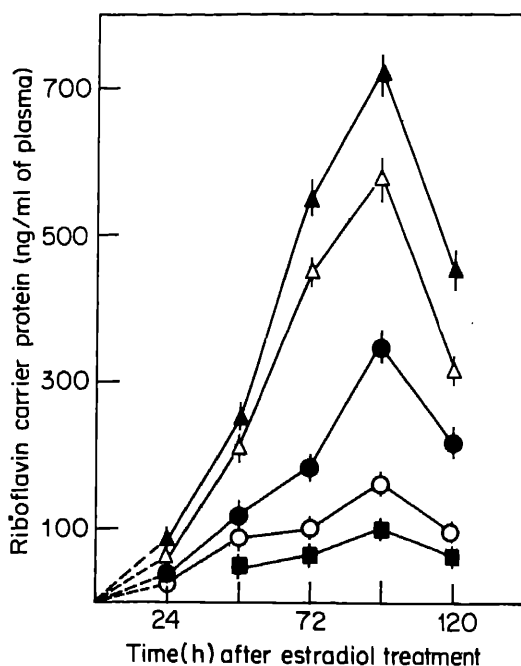
Figure 3. Hormonal dose-dependent reponse of the plasma riboflavin carrier protein during primary stimulation. Time course of plasma riboflavin carrier protein accumulation with increasing concentrations of oestradiol-17β (mg/kg body wt.): ■, 0.1; O, 0.5; ●, 1; △, 5.0; ▲, 10.0. The values represent means and vertical bars represent S.D. (n=5).

Table 1. Effect of administration of cycloheximide on estradiol-induced riboflavin carrier protein accumulation in the plasma.

Treatment (mg/kg body wt)	ng RCP/ml plasma at 72 h
Control	25±8
Estradiol (10)	480±84
Estradiol + cyclohexiimide (0.8)	32±6
Cyclohexiimide (0.8)	29±10

Cycloheximide (0.8 mg/kg body wt) was administered intraperitoneally 30 min prior to injection of estradiol 17β and plasma riboflavin-carrier protein levels estimated at 72 h after estrogenisation. Data expressed as means ±S.D. (n=4).

Effect of progesterone and antiestrogens

Earlier observations with the chicken oviduct system that there exists an antagonism between estrogen and progesterone in terms of ovalbumin induction

during primary stimulation (Palmiter and Wren, 1971) could not be demonstrated with the estrogenized chicken liver system elaborating RCP, thiamine- and biotin-carrier proteins (Murty and Adiga, 1978; Muniyappa and Adiga, 1980a, Ramana Murty and Adiga, unpublished results). It was therefore of interest to investigate whether RCP induction in rat by estradiol is modulated by progesterone administered either alone or in combination with estradiol. From the data of figure 4, it is unequivocally clear that progesterone did not exhibit any discernible influence on the kinetics of estrogen-induced RCP production in the rodent with regard to either the initial lag phase or the subsequent rates of RCP accumulation and decay in the circulation or the peak levels of the protein reached at around 96 h after estradiol treatment.
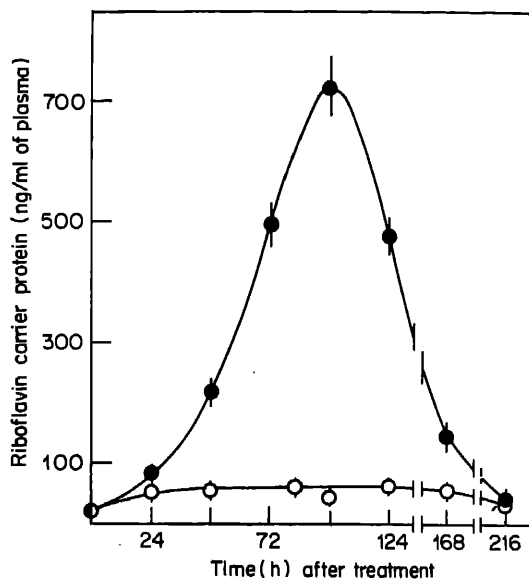
Figure 4. Time course of appearance of riboflavin carrier protein in the plasma on treatment with estradiol-17β (10 mg/kg body wt) + progesterone (10 mg/kg body wt) (●), and progesterone (10 mg/kg body wt) alone (O).

The values represent means and the vertical bars represent S.D. (n=5).

In order to further substantiate this hormonal specificity of RCP induction in the rat, the potent antiestrogens viz., En- and Zu-clomiphene citrates were administered to the animals 30 min prior to the estrogen treatment and the plasma RCP levels were quantified at 72 h. From the results presented in table 2, it is clear that both the antiestrogens have effectively curtailed the estrogenic response besides being ineffective by themselves to elicit even weak estrogen-like activity with regard to RCP induction.

Passive immunoneutralization with homologous antiserum and effect on pregnancy progression in the female rats

The previous observation (Muniyappa and Adiga, 1980b) that RCP is vitally involved in transplacental flavin transport to supply vitamin to the growing

Table 2. Influence of antiestrogens on the estradiol-17β stimulated plasma riboflavin carrier protein content.

Anti estrogens (mg/kg body wt.)	ng RCP/ml plasma at 72 h	
	$-E_2$	$+E_2$
Control (none)	15±6	510±94
E Clomiphene citrate (10)	23±5	58±15
Z-Clomiphene citrate (10)	21±10	69±12

En- and Zu-clomiphene citrates (10 mg/kg body wt) were administered 30 min prior to estradiol-17β administration and plasma riboflavin carrier proteins were estimated at 72 h after estrogenisation. Data are expressed as means ±S.D. (n=4).

foetuses, was based on the observation that passive immunoneutralisation with the antiserum to the chicken RCP led to foetal wastage followed by termination of pregnancy. With the availability of a potent antiserum to the homologous protein, it was desirable to study the influence of neutralization of endogenous RCP by this antiserum on the progression of pregnancy in the rodent. The data of table 3

Table 3. The influence of the specific antiserum to the rat riboflavin carrier protein on pregnant rats.

Treatment	No. of animals	nT/T	Autopsy on day 18	Remarks
NRS treated	5	40/8	40/8	Normal
A/s to ovomucoid	5	48/9.6	47/9.4	Normal
A/s to rat RCP	5	43/8.6	0/0	Slight vaginal bleeding during 24-48 h.
A/s to rat RCP neutralised with rat RCP	5	51/10.2	50/10	No bleeding Normal pups

The pregnant rats (11 day) were treated with either the non-immune rabbit serum (NRS) (0.5 ml), or ovomucoid antiserum or antiserum to the purified rat riboflavin carrier protein raised in rabbits (0.5 ml/rat). nT=total number of implantation sites; T=average implantation sites/ animal determined at laprotomy. The sera were incubated at 52°C for 5 min to inactivate the complement and were administered intraperitoneally. A/S=antiserum.

provide unequivocal evidence that intraperitoneal administration to pregnant rats of adequate quantities of the antiserum to the homologous RCP on day 11 of gestation proved deleterious and resulted in 100% termination of pregnancy within

24-28h after antiserum administration. It was also found that this phenomenon was preceded by slight vaginal bleeding in these animals. In contrast, pregnant animals treated with either non-immunised rabbit serum or with antiserum to a highly immunogenic protein viz. ovomucoid were unaffected in terms of suppression of pregnancy.

Discussion

Consistent with our earlier observations with ovarectomised female rats (Muniyappa and Adiga, 1980b), the data presented above amply substantiate that a single injection of estradiol-17β induces RCP in a dose-dependent manner in immature male rats as well. A comparison of these kinetic data with those obtained earlier with chicken (Murthy and Adiga, 1978) reveal several interesting similarities in the induction of RCP in the two species of animals. Quantitatively, these extend from initial lag phase during which no significant protein accumulates in the plasma, through the patterns of rapid rise and decay in plasma concentrations during primary induction, to significant amplification of the inductive response during secondary stimulation with the steroid. Quantitatively, however, the rodent liver responding to the hormone in terms of RCP induction seems kinetically more sluggish as judged from the relatively protracted initial lag phase (of nearly 24 h compared to 4-5 h in the chicken,(Murthy and Adiga,1978), an extended duration of both ascending and descending phases and relatively lower magnitude of amplification during secondary stimulation. Nonetheless, overall similarity in the pattern of RCP induction in the two species is clearly evident. That the underlying mechanism may also be analogous is supported by the finding that as in the chicken liver (Lazier & Haggarty, 1979), specific, high-affinity cytoplasmic estrogen receptors capable of rapid nuclear translocation and functional interaction with chromatin acceptor sites exist in hepatocytes of both the male and female rats (Atew *et al.*, 1978) and that hormone administration brings about marked changes in the composition and function of the mammalian liver (Seal and Doe, 1969).

The characteristic 'memory effect' with attendant amplification of the inductive response (figure 2) seen during secondary stimulation with the hormonal steroid has also been observed earlier with the estrogenised liver synthesizing RCP (Murthy and Adiga, 1978), TCP (Muniyappa and Adiga, 1980a) biotin binding protein (Ramana Murty and Adiga, unpublished observation) and in chicken (Gruber *et al.*, 1976) and amphibian (Tata, 1976) liver systems elaborating vitellogenin. Though the intricacies of exact molecular mechanism involved need further elucidation, more pronounced transcription presumably due to permanent changes in chromatin (Jost *et al.*, 1978) and rapid translation due to stabilization of mRNAs during this period have been definitely established as dominant parameters responsible for more efficient induction of these proteins. Furthermore, recent investigations with *X. laevis* (Hayward *et al.*, 1981) have shown that enhanced synthesis as well as marked redistribution of available estrogen receptors between nuclear and cytoplasmic components of the hepatocytes with resultant availability of relatively more cytoplasmic receptors for rapid nuclear translocation of the hormone during secondary stimulation may also be an important contributory factor in this regard. Whatever the underlying mechanism, it is clear from the

results of figure 2 that the amplification of estrogen-induced specific gene expression during secondary stimulation is not confined to oviparous species, but extends to mammals also. The only other instance of this kind demonstrated hitherto in mammals is related to enhanced production of prolactin mRNA (but not its translation product) in the pituitary of male rats repeatedly treated with estrogen (Seo *et al.*, 1979). However, it is pertinent to point out that prolactin, unlike RCP, is a normal constituent and secretory product of the pituitary tissue and that the steroid only enhances the expression of its gene and does not induce its synthesis *de novo*.

It is now well documented that estrogen administration brings about the modulation of synthesis of several plasma proteins in the mammalian liver (Seal and Doe, 1969); the plasma renin substrate is notable among these in that its levels can be enhanced by the steroid hormone in a dose-dependent manner in the adult rat though not in prepubertal rats, which have limited population of hepatic estradiol receptors available at this stage (Dickson and Eisenfeld, 1979). The reasons for age-dependant difference in inducibility between plasma renin substrate and RCP are not clear at present. It may be related to the fact that different threshold concentrations of estrogen-receptor complex may be needed at nuclear chromatin acceptor sites for the induction of different proteins (Palmiter, *et al.*, 1981). Based on this premise, it is reasonable to surmise that relatively lower levels of estrogen-receptors available in prepubertal rat liver are sufficient to induce RCP, but not plasma renin substrate. The proof of this hypothesis however awaits further study.

Another intriguing facet of analogy between the rat and the chicken systems with regard to RCP induction is the hormonal specificity as exemplified by complete lack of response to progesterone, either alone or in combination with estrogen (figure 4). Further support for this specificity stems from the data in table 2 regarding complete blockade of RCP induction by anti-estrogens. Again as opposed to the findings with rat uterus (Mohla and Prasad, 1969) these antihormones failed to exhibit any descernible estrogen-like activity by themselves in terms of RCP induction in the rodent liver.

From the foregoing, it is clear that despite marked differences between the rat and the oviparous species in the patterns of embryonic development, the carrier-protein-mediated vitamin delivery mechanism is conserved during evolution of species. The similarity is not only in some structural and functional features of the carrier protein but also with respect to the pattern of hormonal inducibility in the liver. Since the data of table 3 clearly reinforce the earlier postulation that RCP is vitally concerned with embryonic development in both the species (Muniyappa and Adiga, 1980b; Murty and Adiga, 1981), it is clear that estrogen-induced specific protein synthesis in mammalian liver is not merely an evolutionary vestige and that in the female mammal some of these proteins may have an important function during pregnancy as nutrient carriers for embryonic development. Viewed from this angle, it would appear that the mammalian liver, like its avian counterpart (Gruber *et al.*, 1976) can also be considered as an accessory sex organ.

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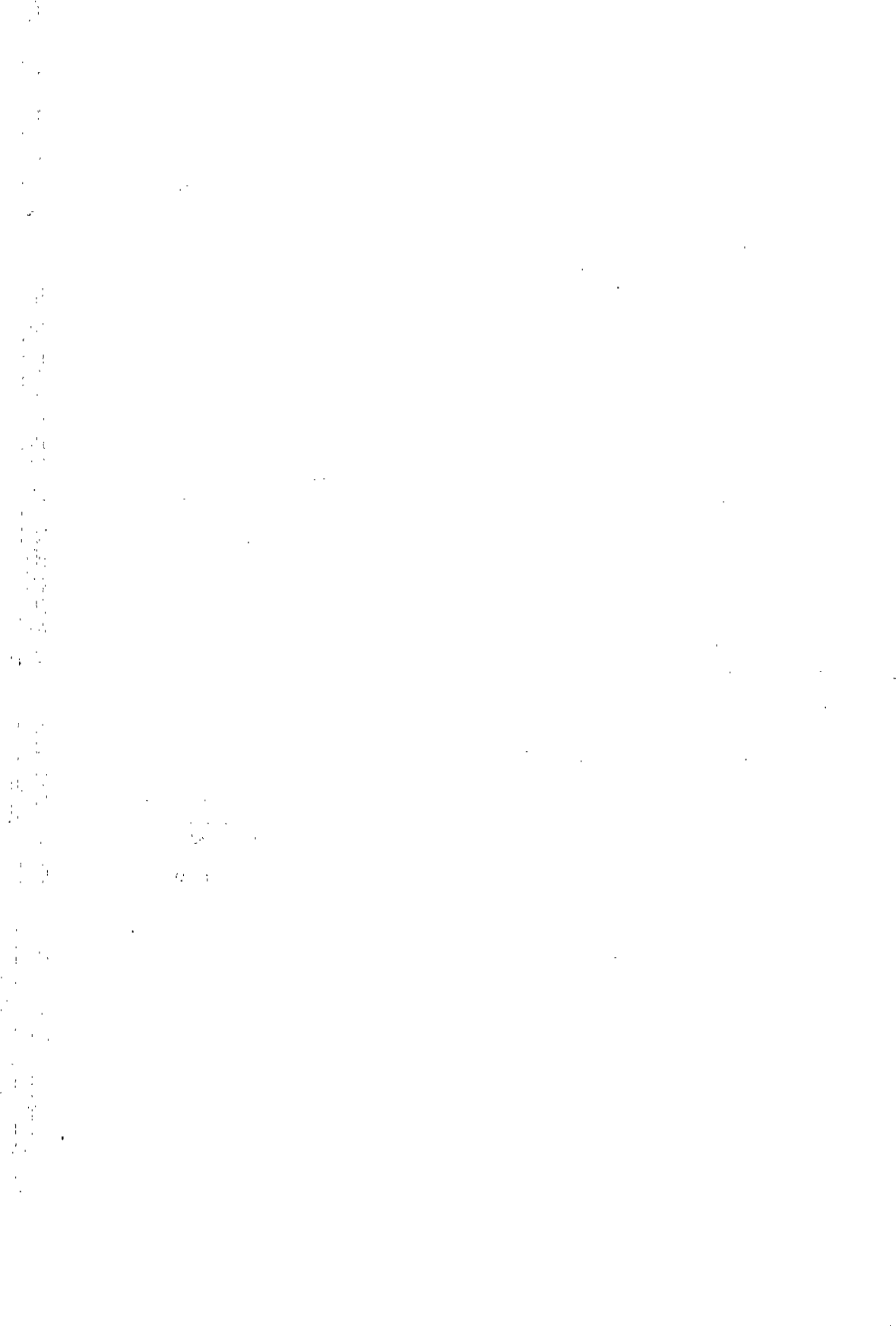
Evidence for non-coordinated synthesis of 5 S RNA in the thermophilic fungus, *Thermomyces lanuginosus*

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Abstract. Analysis of ribosomes and the post ribosomal supernatant fraction of actively growing cells of *Thermomyces lanuginosus* showed the presence of free 5 S RNA in the supernatant fraction. This 5 S RNA was identical to the ribosomal 5 S RNA in its electrophoretic mobility on 10% polyacrylamide gel and in its base composition. 5 S RNA from both the sources gave evidence for the presence of diphosphate at the 5' end. Most of the 5 S RNA that appeared in the cytoplasm was that transported from the nucleus during the isolation. This could be prevented by the use of a hexylene glycol-HEPES buffer.

Keywords. 5 S RNA; thermophilic fungus; ribosomal RNA; non-coordinated synthesis of PNA

Introduction

The genes for 5 S ribosomal RNA of prokaryotes are located on the chromosome as a cluster with other ribosomal RNA components and these RNA species are transcribed together in a unimolar ratio (Kossman *et al.*, 1971; Hayes *et al.*, 1975. On the other hand, eukaryotic 5 S RNA is a primary transcription product and often contains diphosphates or triphosphates at the 5' terminus (Takai *et al.*, 1975). Precursor 5 S RNA species when analysed have been found to have extra sequences at the 3' end but not at the 5' end (Rubin and Hogness, 1975). As the synthesis of eukaryotic 5 S RNA is independent of that of high molecular weight rRNA, an exact 1:1 molar ratio between the two types cannot be expected. In agreement with this, the kinetics of labelling of RNA with [³H]-uridine has shown that 5 S RNA is synthesized in excess in HeLa cells and a free pool of 5 S RNA has been observed in the HeLa Cell nucleus (Knight and Darnell, 1967). It is reported that in exponentially growing HeLa cells the amount of 5 S RNA synthesized is four times greater than the 18 S and 28 S rRNA species (Leibowitz *et al.*, 1973). Seventy five per cent of the 5 S RNA synthesized is apparently degraded. In the ovaries of *Xenopus laevis* non-coordinated accumulation of 5 S RNA in early oogenesis has been observed (Ford, 1971). In this system the relative proportion of 5 S RNA has

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been found to vary widely at different stages of oogenesis. Although there is an excess production of 5 S RNA during early oogensis it is compenstated by an excess production of 18 S and 28 S rRNA at later stages. The presence of free 5 S RNA has been found in the post-ribosomal supernatant fraction of rabbit reticulocytes (Zahavi-Willner and Danon, 1972), but it has been suggested that the soluble 5 S RNA present in reticulocytes arises by the degradation of ribosomes. Thus the production of 5 S RNA in various systems apparently does not follow a uniform pattern. Very little information is available regarding the biosynthesis and the nature of 5 S RNA in thermophilic fungi like *Thermomyces lanuginosus*. In this report we present evidence for the non-coordinated synthesis of 5 S RNA in logarithmically growing cells of *T. lanuginosus* and for the occurrence of a diphosphate at its 5' end.

Materials and methods

Details regarding the organism and sources of radioactive materials and chemicals have been given earlier (Nageswara Rao and Cherayil, 1979).

Preparation of (^{32}P)-labelled RNA from ribosomes and S_{100} fractions

The fungus was labelled with radioactive phosphorus as indicated earlier (Nageswara Rao and Cherayil, 1979). After 18-22 h of growth the mycelia were collected by filtration followed by washing with water and drying. The fungal cake was mixed with glass powder (1 g for 1 g of fungus) and ground for 10-15 minutes at 0°C. The finely ground mycelial paste was extracted with polysomal extraction buffer containing 0.025 M Tris-HCl, pH 7.4, 0.02 M NaCl and 0.005 M MgCl$_2$ (5 ml/g wet weight of the cells). The mixture was centrifuged at 12,000 g for 15 min to remove the glass powder and cell debris. The supernatant fraction was recentrifuged at 105,000 g for 1 h. RNA was prepared from the pellet and supernatant fractions separately.

Instead of Tris-HCl, a buffer consisting of hexylene glycol (0.5 M), HEPES (0.05 mM) and CaCl$_2$ (1.0 mM), pH 6.8 was also used for the preparation of ribosomes and post-ribosomal supernatant fractions.

RNA from the ribosomes and the supernatant fractions was prepared by repeated extraction with phenol. The ribosomal pellet was stirred with 1-2 ml of a buffer containing 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.3, 0.001 M Na$_2$EDTA and 0.5% SDS. An equal volume of water-saturated phenol was added and stirred for 1 h. The aqueous phase was collected by centrifugation and again extracted with phenol two more times. The RNA was precipitated with two volumes of ethanol and washed with 75% ethanol. RNA from post-ribosomal supernatant fraction was prepared in a similar manner by extraction with phenol. The RNA samples thus prepared were subjected to gel-electrophoresis on polyacrylamide gels (Peacock and Dingman, 1967).

Results

^{32}P-Labelled RNA samples isolated from ribosomes as well as S_{100} fraction were subjected to electrophoresis on a 10% acrylamide slab gel (Peacock and Dingman,

1967). Upon autoradiography the RNA sample from ribosomes showed the presence of 5.8 S RNA and 5 S RNA well separated from each other in addition to some contaminating tRNA. There was a considerable amount of radioactivity at the origin due to the high molecular weight rRNA which did not enter the gel. The sample from the post-ribosomal supernatant fraction showed the presence of tRNA and 5 S RNA (figure 1). There was no evidence for the presence of 5.8 S RNA in this fraction and there was very little radioactivity at the origin indicating the absence of contaminating ribosomes. Thus the presence of free 5 S RNA was indicated in the post-ribosomal supernatant fraction. The RNA from both the fractions moved with the same mobility, which was slightly slower than that of xylene cyanol and was similar to that of 5 S RNA from other sources.
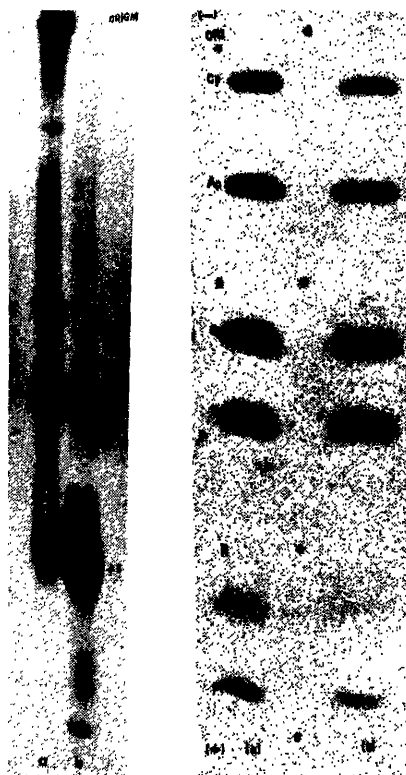
Figure 1. Autoradiogram of the separation of 5 S RNA on a 10% polyacrylamide slab gel.

RNA samples prepared from ribosomes and post-ribosomal supernatant fractions were subjected to electrophoresis on 10% polyacrylamide gels in Tris-borate buffer, pH 8.3 followed by autoradiography. (a) RNA from ribosomes, (b) RNA from post-ribosomal fraction.

Figure 2. Autoradiogram of the separation of RNase T_2 digestion products of 5 S RNA.

The 5 S RNA bands from the ribosomal and post-ribosomal supernatant fractions were cut out from the gel, the radioactivity eluted from each, digested and subjected to electrophoresis on paper at pH 3.5. (a) Ribosomal, (b) Post-ribosomal.

The bands corresponding to 5 S RNA from the ribosomal and post-ribosomal supernatant fractions were cut out separately, and the RNA was eluted from the gel and digested with RNase T_2. The digest was then subjected to electrophoresis on paper at pH 3.5. The pattern of separation of radioactivity was identical in both the cases. In addition to the four major nucleotide species a minor species (denoted as N_p) moving faster than the red dye could be noted (figure 2). No other nucleotide spot could be observed in the autoradiogram. The spots were cut out and the radioactivity in each was determined. Base composition obtained from the data clearly indicated that both the 5 S RNA samples were identical (table 1).

Table 1. Base composition of 5 S RNA from *T. lanuginosus*.

Nucleotide	Ribosomal		Post-ribosomal	
	CPM	%	CPM	%
C_p	9,820	26.3	4,370	25.9
A_p	7,660	20.6	3,480	20.6
G_p	11,080	29.7	4,880	28.9
U_p	7,810	20.9	3,720	22.0
pp^Gp	940	2.5	440	2.6

The 5 S RNA bands were cut out from the gel and the radioactivity from each band was extracted. It was digested with RNase T_2 and nucleotides were separated by high-voltage paper electrophoresis at pH 3.5 in pyridine-acetate buffer. After auto-radiography (figure 2) the radioactive spots were cut out and counted in a liquid scintillation counter. The results of one of the representative experiments are presented.

The radioactivity in the unknown spot amounted to approximately 2.5% of the total, representing 3 phosphate groups for a chain of 120 nucleotides of the 5 S RNA (Erdmann, 1977). Therefore the minor spot could be a modified mononucleotide in the ratio of 3 mol per mol of 5 S RNA, an alkali resistant O'-methylated trinucleotide in unimolar ratio or a nucleotide with three phosphate groups on it. Since no 5 S RNA has so far been reported to contain modified nucleotides, except submolar amounts of pseudouridine, the possibility that the minor spot might be a nucleoside with three phosphate groups appeared more likely. Obviously, the nucleotide being an RNase T_2 digestion product, must be of the type ppNp, released from the 5' end. The spot moved faster than pGp and pppG which have 4 units of negative charge each, although pppG has an extra phosphate group. It moved very much faster than pAp and pCp' both of which have the same mobility as Up. pUp on the other hand, moved faster than the unknown spot. When considered among the various possible ppNp structures, ppGp was the most probable 5' end of the 5 S RNA molecule. This type of 5' end has been reported for 5 S RNA from a number of sources such as different strains of yeast (Erdmann, 1977).

It was not clear from the above studies whether the 5 S RNA observed in the cytoplasm was originally present there or whether it leached out into the cytoplasm from the nucleus during the isolation of the S_{100} fraction. A buffer system consisting of hexylene glycol and HEPES is known to prevent the leaching out of nuclear components into the cytoplasm (Wray *et al.*, 1977). When post-ribosomal supernatant fraction was prepared using hexylene-glycol-HEPES buffer, pH 6.8, there was practically very little 5 S RNA in the supernatant fraction (Results not presented). This indicated that the free 5 S RNA largely remained in the nucleus during the growth of the cell.

Discussion

It is evident from the present studies that there is a pool of soluble 5 S RNA (unassociated with proteins) in the nucleus of *T. lanuginosus* at the log phase of its growth. This free 5 S RNA could leach out during extraction with aqueous buffers normally used. The pool size in the present case is not known. The results clearly indicate that in *T. lanuginosus* 5 S RNA is synthesized in amounts larger than that required for the maintenance of a stoichiometric ratio with other ribosomal species. Although an excess of 5 S RNA is produced in early oogenesis in *Xenopus*, it is compensated by an excess production of 18 S and 28 S RNAs later (Ford, 1971). In HeLa cells the excess 5 S RNA produced is degraded (Leibowitz *et al.*, 1973). As there is an excess production of 5 S RNA in actively growing *T. lanuginosus* cells, in which ribosome synthesis is expected to take place at a very fast rate, it must be assumed that there is no compensation. The excess of 5 S RNA produced may be degraded.

Transport of soluble nuclear components into the cytoplasm from the nucleus during extraction has been observed in a number of cases. This is restricted mainly to low molecular weight components. Presence of precursors of tRNA in the cytoplasm has been noted, although the splicing and processing enzymes are present in the nucleus only (Melton *et al.*, 1980). The present studies have shown that migration of 5 S RNA from the nucleus to the cytoplasm may be prevented by the use of the hexylene-glycol-HEPES buffer. This may apply to precursors of tRNA also. Under the conditions employed the nucleus apparently is not disrupted. No RNA, other than 5 S RNA and tRNA is observed in the cytoplasm.

The presence of a small amount of 5 S RNA even when hexylene-glycol-HEPES buffer is used has been noted. It is not clear whether a small amount of 5 S RNA was transported into the cytoplasm during the isolation or whether it was originally present there *in vivo*. It has been reported by Wreschner (1978) that 5 S RNA in nanogram quantities inhibits the translation of globin mRNA in wheat germ system whereas other RNAs tested do not. It is possible that the free 5 S RNA acts as a modulator of protein synthesis. It is conceivable that the free 5 S RNA present in the nucleus can, under certain metabolic conditions, cross over into the cytoplasm and exert its inhibitory influence on protein synthesis and thus control translation.

Eukaryotic 5 S RNA is a primary transcription product and in most cases it has a triphosphate at the 5' end (Erdmann, 1977). The 5 S RNA of *T. lanuginosus*, on the

other hand, has a diphosphate at the 5' end. In this respect it resembles yeast, to which it is related. The mechanism of processing of the triphosphate to diphosphate and the significance of the presence of the diphosphate at the 5' end of the mature 5 S RNA is not yet clear.

Acknowledgement

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Notes on the preparation of papers

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Structural and functional changes in the intestine of irradiated and hypothermic irradiated rats: A scanning and transmission electron microscopic study

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Abstract. Severe destructive changes in the intestine of rats following whole body exposure to gamma rays (832 rads) were observed by light microscope, scanning and transmission electron microscope studies. Hypothermia (15°C rectal temperature) induced prior to irradiation protected the intestinal mucosa from destruction. A simultaneous study showed that glucose absorption decreased significantly in irradiated rats, whereas it was increased in hypothermic irradiated animals.

Keywords. Gamma irradiation; transmission electron microscope; scanning electron microscope; glucose absorption; gastro-intestinal syndrome.

Introduction

Radiation induced destruction of intestinal mucosa is well documented (Hageman *et al.*, 1961; Patt and Quastler, 1963). Intestinal functions, for example, the transport of glucose and other nutrient materials were depressed in addition to cellular damage during the initial period of irradiaton (Perris *et al.*, 1966; Perris, 1966; Altman *et al.*, 1970). These reports suggest a possible dependence of intestinal absorptive function upon its architecture. The above information on morphology of intestine exposed to radiation is based largely on histological studies using light microscope (Quastler and Hampton, 1962; Detric *et al.*, 1963; Hugon and Borgers, 1963; Wiernik and Pant, 1970). It was, therefore, of interest to examine the details of structural destruction of the intestinal mucosa at the ultrastructural as well as at light microscope level and to correlate with a biochemical function such as glucose absorption. Whole-body radiation which is a metabolic depressant and has destructive effects on intestinal mucosa, and hypothermia, which acts as a radioprotectant and metabolic depressant like radiations, constitute valuable experimental tools for studying such structure-function relationship of intestinal mucosa. An attempt was, therefore, made in the present investigation to study as to what extent passive (energy independent) and active (energy dependent) transport of especially glucose in intestine is dependent on the normal structure of the intestinal mucosa.

Materials and methods

A total of 60 adult rats (Charles Foster strain) of both sexes (100-150 g) were used in the present investigation and grouped as: (I) Normal sham irradiated controls

(N), (II) irradiated, observed at 24 h (I-24), (III) Hypothermic-irradiated (HI-24) and (IV) Hypothermic control (H-24) using 15 rats in each group. All the animals were fasted for a period of 18 h before they were subjected to radiation or hypothermia. Animals of group II were then exposed to whole body gamma radiation of 832 rads from a telecaesium source (Cs-137) the dose rate being 70 rads/min. Hypothermia was induced in the animals of groups (III) and (IV) at the 17th h of fasting by packing ice over the abdominal surface after light ether anaesthesia, cooled to a rectal temperature of 15°C and maintained at that temperature for a period of 1 h. Hypothermia in group III animals was maintained during the total period of radiation exposure. Animals of group III were subjected to irradiation as described in group II animals and those of group IV were left as such for normal recovery. All the animals excepting those maintained for glucose absorption studies were sacrificed at 24 h and portions of intestine were removed for histological, scanning and transmission electron microscopic studies and mucosal DNA estimation.

Histological techniques

A portion of the duodenum was fixed overnight in 4% formaldehyde. Paraffin sections were cut at 5 μ thickness. Deparaffinized sections were then stained by 'De Thomasi Schiff Reagent (Feulgen method) and counterstained by 1% aqueous light green (Pearse, 1960). Villi height and crypt lengths were measured under light microscope using eyepiece micrometer.

Preparations of specimen for scanning electron microscopy

Small lengths of the intestine were cut longitudinally avoiding injury to the mucosal surface and stretched over flat pieces of cork with the mucosal surface facing up. Mucosal surface was washed with normal saline, fixed immediately in 2% glutaradehyde in phosphate buffer for 4 h and following several washes in buffer the tissues were post-fixed in 1% aqueous osmium tetroxide for 1 h. Tissues were next washed several times with water. They were then dehydrated in graded alcohol and dried in a vaccum desicator. About 5 mm square pieces were mounted over the specimen carrier. The surface of the tissues was coated with a conducting layer of gold pellets and examined under scanning electron microscope (Philips SEM 500).

Preparations of ultrathin sections for transmission electron microscopy

Small portions of the duodenum (0.5 cm) were quickly taken out and split longitudinally with fine pair of scissors avoiding injury to the mucosal surface.

They were then immediately fixed in 1% buffered (pH 7.2 to 7.5) glutaraldehyde solutions for 2 h followed by several washes in Sorensen phosphate buffer. These were then post-fixed in 1% veronal buffered osmium tetroxide for 4 h. After repeated washes in veronal buffer the tissues were cut in small blocks, measuring 1-2 cmm, at right angles to the mucosal surface. Following dehydration and clearing, the tissues were embedded in Epon 812 with proper orientation so as to get sections along the long axis of the villus. Thin serial sections were cut in a Porter Blum ultramicrotome and finally mounted on 300 mesh copper grids. These were

then stained with 1% uranyl acetate follwed by lead citrate (Reynolds, 1963). The sections were finally examined using Seimen's Elveiscope Model I Electron-microscope.

Mucosal DNA estimation

Weighed quantities of mucosal scrappings from the intestine of the four groups of rats were digested in 5% perchloric acid and finally colour was developed by the diphenylamine reaction (Plummer, 1971).

Glucose absorption

Glucose absorption was studied in all the above groups of animals at 24 h using a method similar to that described by Cori (1925); 2.5 ml of 50% glucose solution (containing 1250 mg of glucose) warmed to 38°C was administered to each animal orally with the help of a stomach tube. Two hours later the residual glucose in the gastrointestinal tract was estimated by the method of King and Asatoor (1954). The difference between the amount administered and the amount left in the gastro-intestinal tract gives a measure of the amount absorbed.

Results

In the control group of rats (group I), duodenal mucosa consisted of tall finger like villi having a core of lamina propria and lined by densely packed tall columnar cells with intact basement membrane; tip denudation was infrequently seen with proliferative (2-3 mitotic figures/crypt) crypts in regular continuity with the villi. The mean villus height and crypt length as measured were 4447 ± 44.7 μ and 158.8 ± 15.1 μ respectively (table 1). In the irradiated animals (group II) measurement of

Table 1. Mean villi height, crypt length, mitotic rate and the corresponding DNA values in the intestinal mucosa of normal (control), hypothermic-irradiated and hypothermic (control) rats.

Groups	Mean villi height (μ) ±S.D.	Mean crypt length (μ) ±S.D.	Mean mitotic rate mitoses/ crypt.	DNA content of intestinal mucosa μg/mg dry weight Mean ±S.D.	Significance between (DNA content)
Normal control (N)	447.7 ±46.7	158.8 ±15.1	2.25	48.26 ±2.06	N and I-24 $t = 7.23. p < 0.001$
Irradiated 24 h (I-24)	N.D.*	N.D.*	0	28.05 ±7.60	I-24 and HI-24 $t = 10.74. p < 0.001$
Hypothermic irradiated- 24 h (HI-24)	671.3 ±64.9	180.4 ±28.5	0.66	57.76 ±1.73	N and HI-24 $t = 13.27. p < 0.001$
Hypothermic control (24 h) H-24	641.8 ±39.4	173.0 ±19.0	0.95	50.93 ±2.59	

* Could not be measured due to widespread destruction.

villus height posed a problem due to widespread destruction; they were often found as stunted, clumped like structure due to adhesion between different villi. The cells with picnotic nuclei and evidences of cellular destruction could be seen. Lining cells were found disorganised, less densely packed with frequent disruptions in the basement membrane. Crypts were found shrunken, reduced in size, lined by single layer of cells and filled with cellular debris with complete absence of proliferative activity (figure 1A). In hypothermic irradiated rats (group III) the duodenal mucosa showed long finger shaped villi measuring 671.3 ± 64.9 µ with no evidence of damage (figure 1B). Cell denudation at villus tips was infrequent and majority showed a club-shaped appearance. The lining cells were found well organised and well preserved. The mean crypt length as measured was 180.4 ± 28.5 µ. Proliferative activity in the crypts could be seen at places (0.66 mitoses/crypt). A hypothermic control group of rats also showed similar features (table 1).

Figure 1. A and B: Histological feature of duodenum showing villi height and crypt length in (A) irradiated (I-24) and (B) Hypothermic irradiated (HI-24) rat (magnification: 100 × 2.5).

Mucosal DNA content

DNA content of the intestinal mucosa (table 1) in irradiated rats was found less (28.05 ± 7.00 µg/mg dry weight) than in controls rats (48.26 ± 2.08 µg/mg dry weight): in hypothermic irradiated and hypothermic control groups it was increased to 57.76 ± 1.73 µg/mg dry weight, and 50.93 ± 2.56 µg/mg dry weight respectively.

Surface ultrastructure by scanning electron microscopy

Under scanning electron microscopy the normal villi appeared as regularly arranged structures with intact tip head (figure 2A, 2B). The surface of each villus showed discontinuous clefts at places giving it a corrugated appearance in the histological sections. At higher magnification (× 800), the enterocytes had polygonal appearance with flat top having convexity upwards. The compact cell arrangement almost assumed a honeycomb' like appearance. The mean surface area of the cells and the mean cell diameter (D) assuming the outline to be circular

Figure 2. A and B: Scanning electron micrograph of intestinal (duodenal) villi of normal rats (N) showing (A) regularly arranged villi and crypts and (B) unbroken villi tips with prominent enterocytes and mucigen secretion from goblet cells (magnification: A, × 600; B, × 4800).

(table 2) were found to be $56.5 \pm 7.1 \, \mu^2$ and $8.5 \pm 0.5 \, \mu$ respectively. The outline of discrete microvillus tips over the enterocyte surface under the Scanning electron microscopy at this magnification could not, however, be made out clearly.

In irradiated rats villi and crypt structures were found disorganised, mostly broken and irregularly arranged. Loss of continuity between the villi and crypts was conspicuous (figure 3A). The villi tips appeared to be tortuous and fragile in most places with evidences of stickiness between them. At higher magnification (× 1600) the villus surface was found severely damaged with either loss of outline (figure 3B) or distortion in shape of individual enterocyte. Their surface area and mean diameter were found reduced significantly (table 2). Micro-villi could not be seen due to the presence of glycocalyx (figure 3B).
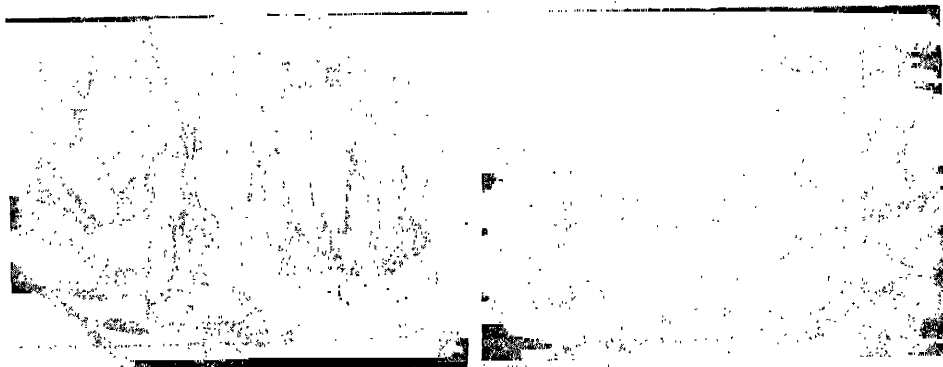
Figure 3. A and B: Scanning electron micrograph of duodenal villi of irradiated (I-24) rat showing (A) broken villi-crypt architecture with (B) disintegrated tip and disorganised lining cells with interrupted basement membrane are more prominent (magnification: A, × 600; B, × 1600).

Table 2. Epithilial cell (enterocyte) dimension as revealed by scanning and transmission electron microscopy.

	Scanning electron microscopy		Transmission electron microscopy	
Groups	Mean enterocytes diameter ± S.D. (μ)	Mean surface area of enterocyte ± S.D. (μ^2)	Mean microvilli height ± S.D. (μ)	Microvilli density: villi/ surface area ± S.D.
Normal (N)	8.5 ± 0.5 t = 10.98; p<0.001 (N and I-24)	56.5 ± 7.1 t = 11.25; p<0.001 (N and I-24)	± 1.02 ± 0.09 t = 11.17; p<0.001 (N and I-24)	50 ± 11
Irradiated 24 h (I-24)	4.7 ± 1.2 t = 11.45; p<0.001 (I-24 and HI-24)	18.9 ± 10.3 t = 9.03; p<0.001 (I-24 and HI-24)	0.63 ± 0.07 t = 30.86; p<0.001	Not measurable due to widespread disruption at places.
Hypothermic irradiated (24 h) (HI-24)	10.6 ± 1.57 t = 4.94; p<0.001 (N and HI-24)	91.2 ± 29.1 t = 4.48; p<0.001 (N and HI-24)	1.84 ± 0.1 t = 11.23; p<0.001 (N and HI-24)	80 ± 12

In contrast, intestinal villi of hypothermic-irradiated rats under scanning electron microscopy were found as tall, smooth, regularly arranged structures. No evidence of damage of crypts could be seen (figure 4A). Villi tips which were mostly intact were found to be more flat and broad. At still higher magnification ($\times$ 4800) compact network of enterocytes with flat-top could be seen with regular outlines. Microvilli appeared as compact structures in regular short-ranged hexagonal array (figure 4B). Significant increases in surface area and diameter of enterocytes were seen (table 2). Hypothermic control animals showed similar changes in cell surface area.
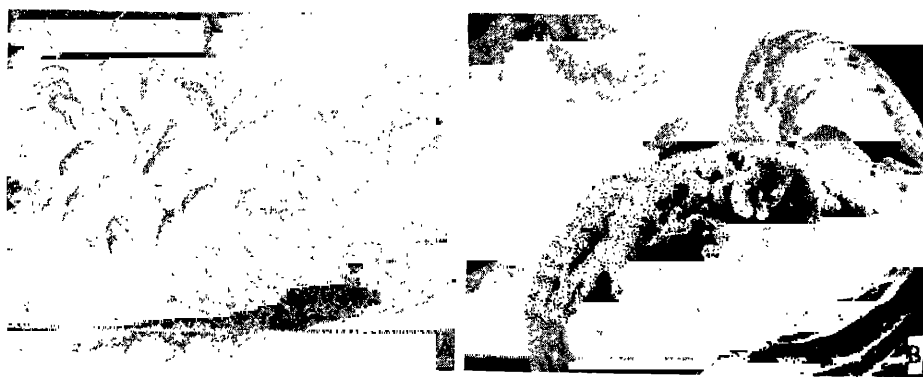
Figure 4. A and B: SEM feature of duodenal villi of hypothermic irradiated (HI-24) rats showing (A) tall, smooth well organised villi-crypt structure and (B) at higher magnification compact network of flat top cells with regular outline (magnification: A, $\times$ 600; B, $\times$ 4800).

Ultrastructure as revealed by transmission electron microscopy

Under transmission electron microscope besides the cytoplasmic structure like mitochondria, ribosomes, endoplasmic reticulum, lysosomal bodies, etc., the epithelial brush border or microvilli of individual enterocyte could be seen (figure 5A) and were found to have a mean length of 1.02 ±0.09 µ (table 2). The arrangement of the microvilli were found to be very regular with almost uniform diameter and well defined terminal web area.

Figure 5. A, B and C: Transmission electron micrograph of (A) normal (N), (B) irradiated (I-24) and hypothermic-irradiated (HI-24) rats showing the microvilli arrangement (magnification: A, × 20000; B, × 20000; C, × 20000).

In contrast to the normal, brush border of villus enterocytes of irradiated rat was found to be highly disorganised, relatively short, frequently interrupted and terminal web area indistinct. The mean length of microvilli was of the order of 0.63 ±0.07 μ (figure 5B, table 2). Abnormalities in cytoplasmic structures were also observed, namely, mitochondria were found swollen and less electron dense, lysosomal bodies relatively enlarged.

In the hypothermic irradiated rats, brush border was found relatively tall, well defined, and compact. The length of individual microvilli as measured was found almost doubled (table 2); they were more electron dense, regularly arranged and evenly spaced. The terminal web area could be seen clearly. Mitochondrial swelling was present (figure 5C). Similar changes were observed in hypothermic controls.

Glucose absorption

A large amount of residual sugar was found in the intestine of irradiated animals in contrast to that in normal controls (table 3). Consequently, the total amount of sugar absorbed was significantly less in irradiated animals compared to that of the normal controls. When expressed as percentage of the amount of sugar transported to the intestine by the stomach, the absorption was 56.4% in irradiated and 95.1% in normal control rats respectively (table 3).

Table 3. Intestinal glucose absorption study in normal (N), irradiated (I-24), hypothermic-irradiated (HI-24) and hypothermic control (H-24) rats.

Groups	Glucose administered (mg)	Glucose content in stomach oesophagous (mg)	Glucose presented to intestine (mg)	Glucose absorbed (mg)	% Absorption of glucose as presented to intestine
Normal control (N)	1250	811.3 ±38.7	438.7 ±38.7	417.2 ±32.3	95.18 t= 10.95, P<0.001 (N and I-24)
Irradiated (I-24)	1250	796.1 ±31.3	453.9 ±31.3	256.2 ±23.1	56.40 t= 23.89, p<0.001 (I-24) and HI-24)
Hypothermic Irradiated (HI-24)	1250	729.1 ±25.9	520.9 ±25.9	509.4 ±20.2	97.85 t= 7.65, p<0.001 (N and HI-24)
Hypothermic control Control (HI-24)	1250	619.7 ±27.3	630.3 ±27.3	626.2 ±31.6	99.33

In hypothermic irradiated rats, relatively lesser amount of sugar was left in the stomach as well as intestine indicating a greater absorption by the intestine the percentage absorption as transported to intestine from stomach being 97.8% (table 3). Likewise, the amount absorbed in hypothermic control rats was 99.3% (table 3).

Discussion

The widespread structural destruction observed at 24 h in irradiated rats is perhaps the cause of damage and destruction to intestinal absorptive surface area. The destruction involves both villi and crypt structures (figure 1B). The crypt represents the cell renewal system of intestine and as such becomes functionally depressed as per the law of Bergonie and Tribondeau (1906). The absorptive surface area following exposure to radiation becomes greatly reduced and remains so until the cell renewal system resumes its function. The reduction in absorptive surface is due to (1) destruction of villi and shortening of villi heights (figure 1B), (2) fusion of adjacent villi, (3) loss of lining cells also confirmed by mucosal DNA estimation (table 1), (4) reduction in diameter and surface area of individual enterocytes (table 2) and (5) destructive changes and shortening of microvilli (figure 5B, table 2).

Radiation induced cell loss has been reported by many (Hageman *et al.*, 1961; Patt and Quastler, 1963). The decrease in cell density in intestinal mucosa in irradiated animals is perhaps a combined effect of increased cell loss from villi, and decreased cell proliferation due to inhibition of crypt proliferative activity (table 1). Thus increased villi cell denudation rate and simultaneous depression of crypt cell proliferation rate may be responsible for deranging the homeostasis of the cell renewal system reflected in stunting of villi. Cell loss and associated destructive changes are due to well recognised effects of toxic radicals of radiation (Altman *et al.*, 1970).

Measurement and observation of the microvilli under transmission and scanning electron microscope of individual enterocyte show that normally they are arranged in crystalline hexagonal array. These are supposed to increase the epical surface area of individual cell by a factor of 24 (Palay and Karlin, 1959). The widespread destruction and reduction in height of microvilli (table 2) as seen under transmission electron microscopy in irradiated animals further decreases the total absorptive surface. Such information however, could not be obtained by scanning electron microsope as they are obscured by glycocalyx.

When hypothermia was induced in animals prior to irradiation all the components constituting the absorptive surfaces are preserved in such a way that nearly a 2 fold increase in the absorptive surface takes place. This increase in absorptive surface is contributed by (1) increased heights, thickness and number of intact villi, (2) absence of fusion between adjacent villi, (3) increase in the number of lining cells, (4) increase in the diameter and surface area of individual enterocytes and (5) increases in the length of microvilli. When analysed with the results obtained in hypothermic control animals the above may be considered as to be the direct effects of hypothermia.

Decrease in villi cell denudation rate, tall and broad villi, absence of cellular debris in the intestinal content, and increase in mucosal DNA content in the presence of non-proliferative crypt epithelium are evidences of protection offered by hypothermia and may be explained on the basis of decreased intestinal motility, decreased floral activity depressed cell metabolism and decrease in toxic radical formation during hypothermia. Decrease in the crypt cell proliferation rate during cold, according to Bergonie's Law (1906) renders the crypt cells less radiosensitive. This keeps them ready to renew to their proliferative activity immediately the effects of hypothermia ceases to operate. The reason for (1) increase in the individual cell surface area and (2) nearly two fold increase in enterocyte microvilli thickness in cold are still not very clearly understood.

A significant ($p < 0.001$) decrease in absorption of glucose was observed in irradiated animals. This decrease in absorption is probably due to the structural deformity and destruction of the intestinal mucosa. An interdependence of mucosal cell population and absorptive power of intestine has been demonstrated · by comparing the extent of absorption in coeliac syndrome (flag jejunum) with that of a normal individual (Creamer, 1967). Such interrelation between absorption and mucosal cell population existed between the abnormal mucosa of population of many Asian and African countries and the normal tall shaped appearance of mucosal villi in European and American population (Creamer, 1967).

The above interrelationship can also be demonstrated if the absorptive surface area of intestine is increased above normal. This can be done conveniently by inducing hypothermia in normal or even in irradiated animals as achieved in the present investigation. As a result glucose absorption increased significantly above these in irradiated and normal animals (table 3). In presence of depressed energy metabolism of hypothermia and irradiation, the increased absorptive surface precisely accounts for the above increase in glucose absorption in these two group of animals (Chaudhuri *et al.*, 1979).

It may be observed from the above that (1) under normal metabolic state glucose absorption may remain unaffected only if the extent of absorptive surface is unchanged, (2) under depressed metabolic state normal or any reduction in absorptive surface will result in a decrease in glucose absorption and (3) under depressed metabolic state glucose absorption may be normal or even above normal if the absorptive surface is increased to normal or above normal state. Based on these observations it can be postulated that normally both the active and passive transport mechanism determine the absorptive process. Under depressed metabolic state the passive transport is more important than active transport to maintain normal absorption provided adequate absorptive surface is available. Under conditions of reduced area of absorption, active transport will dominate the absorptive process.

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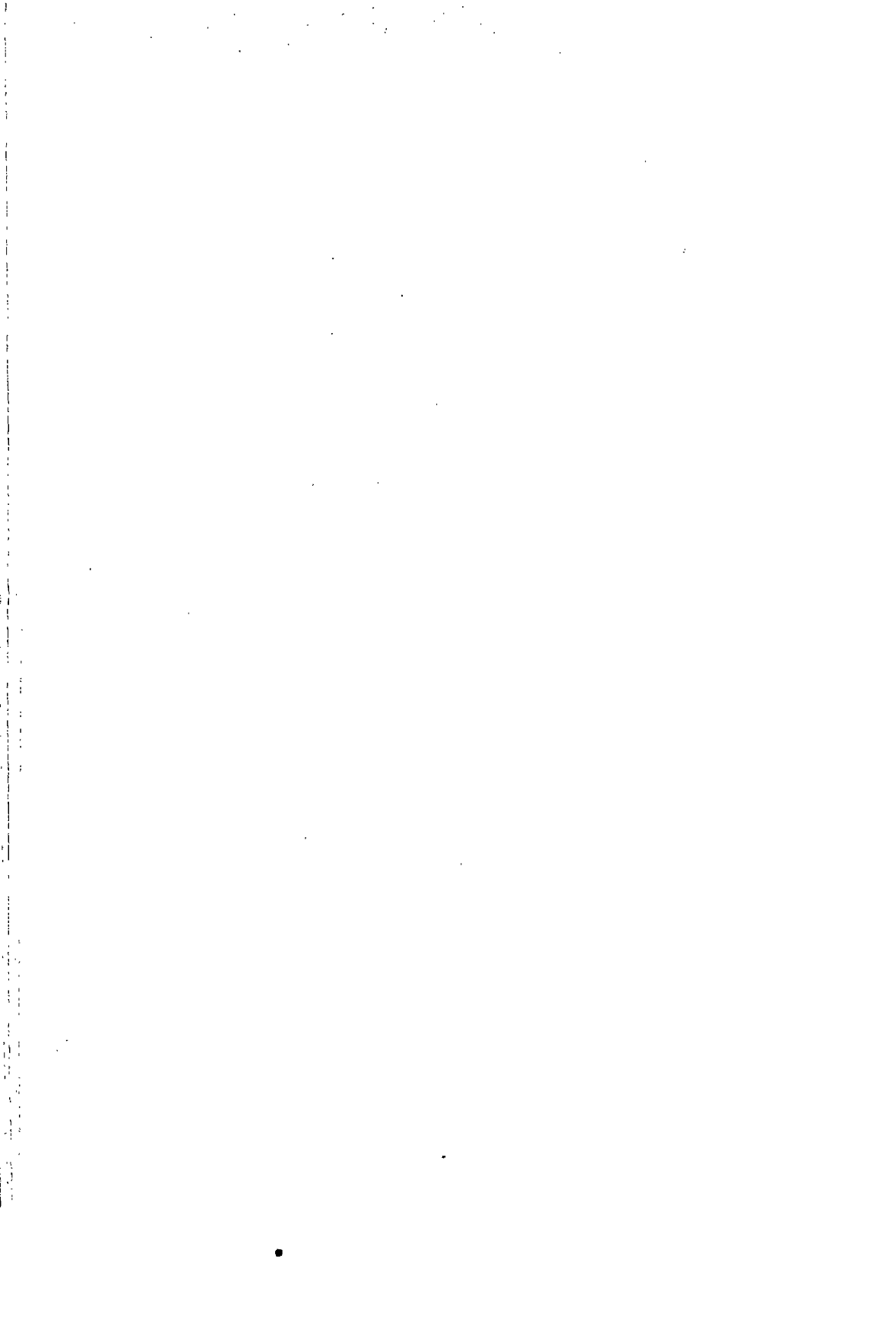
α-D-Galactose-specific lectin from jack fruit *(Artocarpus integra)* seed

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Abstract. An α-D-galactose-specific lectin from the seeds of jack fruit *(Artocarpus integra)* has been isolated in pure form by affinity chromatography on immobilised guar gum (a galactomannan). The lectin is shown to be a glycoprotein containing 3% carbohydrate and having a molecular weight of 39,500 as determined by gel filtration. Sodium dodecyl sulphate gel electrophoresis revealed a single polypeptide of 10,500 dalton, indicating that the native lectin is a tetramer of identical subunits. The hemagglutinating activity of the lectin towards erythrocytes of all blood groups is found to be the same.

Keywords. Jack fruit seed; lectin; α-D-galactose; guar gum; affinity chromatography.

Introduction

Lectins are proteins of non-immune origin, present in plant extracts and they exhibit cell agglutinating property. They are widely distributed in nature, being found in plants, micro-organisms and animals. They bind mono- and oligosaccharides with remarkable specificity in the same way as the enzmes bind substrates and antibodies bind antigens. Although the physiological function of plant and animal lectins is unknown, these ubiquitous carbohydrate-binding (glyco) proteins can recognise and bind to complex carbohydrates as they occur in solution or on membranes and cell surfaces (Lis and Sharon, 1973; Goldstein and Hayes, 1978). There is increasing indication that they function in both intercellular and intracellular recognition phenomena in microorganisms, plants and animals (Boyd, 1970; Goldstein *et al.*, 1968; Simpson *et al.*, 1978). This laboratory has been investigating this important class of proteins present in the locally available seeds, and the purification of N-acetyl-D-galactosamine-specific lectin from winged bean has been reported (Appukuttan and Basu, 1981). In this paper we describe the purification and physicochemical properties of an α-galactose-specific lectin from jack fruit seed *(Artocarpus integra)*. Jack fruits are typical tropical seasonal fruits. Their seeds contain very little fat but contain about 40% carbohydrates and 7% protein.

Abbreviations used: Con A, Concanavalin A; PBS, 20 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl.

Materials and methods

Jack fruit seeds were collected from locally available sources. Crystalline bovine serum albumin, guar gum, Tris, acrylamide, N,N,N′N′-tetramethylene diamine, N,N-methylene bis acrylamide, sodium dodecyl sulphate, 2-mercaptoethanol, Coomassie Brilliant Blue and α-methyl glucoside were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The molecular weight standards were purchased from Pierce Chemical Co., Rockwell, Illinois, USA. Biogel P-100 of Bio-Rad was a gift from Dr Krishna Bakshi of the National Institutes of Health, Bethesda, Maryland, USA. Sepharose 4B and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, *p*-Nitrophenyl derivatives of α-D-galactose and β-D-galactose were purchased from Koch-Light Laboratories, Colnbrook, England. All other chemicals used were Analytical reagent grade. Blood samples were obtained from our Institute's Blood Bank.

Guar gum was insolubilized by cross-linking with epichlorohydrine in alkaline medium according to Appukuttan *et al.* (1977). The protein was estimated with crystalline bovine serum albumin as standard according to the method of Lowry *et al.* (1951). Concanavalin A (ConA) was isolated by the method of Surolia *et al.* (1973). The Con A was immobilized on CNBr-activated Sepharose 4B by the method of Bishayee and Bachhawat (1974).

Polyacrylamide gel electrophoresis was done according to Davis (1964). SDS-polyacrylamide gel electrophoresis was carried out at pH 7.0 (Weber and Osborn, 1969). The molecular weight of native protein was estimated by gel filtration on Biogel P-100 according to Andrews (1965). Neutral sugar was estimated by the phenolsulphuric acid method of Dubois *et al.* (1956).

Purification of jack fruit seed agglutinin

All operations were carried out at 0-4°C unless otherwise mentioned. Dehusked seeds (10 g) were soaked for 24 h in 20 mM potassium phosphate buffer, pH 7.4 containing 150 mM NaCl (PBS). The seeds were cut into small pieces and homogenised with 70 ml of PBS in a Sorvall omnimixer for 3 min at maximum speed. The homogenate was stirred for 3 h and centrifuged at 20,000 g for 15 min. The precipitate was discarded and the supernatant fluid made to 70% saturation (49 g/100 ml) with solid ammonium sulphate. The suspension was stirred for 60 min and the precipitate was collected by centrifugation as before. The ammonium sulphate precipitate was dissolved in 10 ml of PBS and dialysed with 2 changes against the same buffer for 16 h. Any precipitate formed during dialysis was discarded by centrifugation as before.

The dialysed supernatant was absorbed on a cross-linked guar gum column (2×15 cm). The column was equilibrated with PBS and washed with the same buffer till the absorbance at 280 nm of the effluent was lower than 0.05. The agglutinin was then eluted with PBS containing 150 mM galactose and 10 ml fractions were collected. The active fractions (No. 30 to 36) were pooled and dialysed against PBS with several changes for 24 h.

Results and discussion

The cross-linked guar gum retained a portion of the ammonium sulphate-precipitate, which was eluted only with galactose (figure 1). The binding capacity of
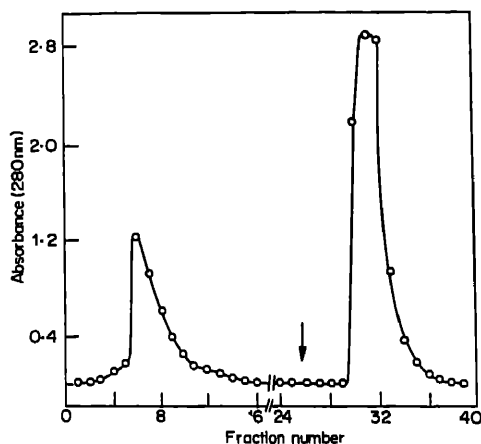
Figure 1. Affinity chromatography of jack fruit seed lectin on cross-linked guar gum. Details are given under materials and methods.

the matrix was found to be 5 mg lectin protein per ml of packed column. More than 50% of the applied protein was found to constitute the lectin. In a separate experiment, the lectin content was found to be 1.2% (by weight) of the seed. The galactose-specific eluate of the protein in disc gel electrophoresis showed a single band at pH 4.5 (figure 2). The protein failed to move at alkaline pH at 5%, 7.5% and 10% acrylamide concentrations.
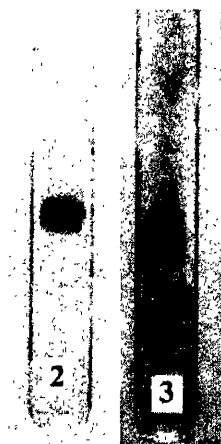
Figure 2. Polyacrylamide gel electrophoresis of purified jack fruit seed lectin at pH 4.5. 30 μg protein was used in 7.5% acrylamide gel.

Figure 3. SDS-polyacrylamide gel electrophoresis of jack fruit seed lectin. 30 μg protein was used in 10% acrylamide gel.

The remarkable capacity of guar gum, a galactomannan to bind α-galactose-specific lectin was demonstrated by the isolation of *Ricinus communis* lectins (Appukuttan *et al.*, 1977). Guar gum contained β-(1→4) linked mannose units in the main chain with a single galactose moiety linked α-(1→6) to every alternate mannose unit (Dea and Morrison, 1975). Due to this structure it has 100-fold increased capacity to bind *Ricinus communis* lectin compared to Sepharose (Appukuttan *et al.*, 1977). The reason for the high binding capacity with the present lectin may be the same. A comparison between the binding affinities of α- and β-linked galactose moieties of *p*-nitrophenyl derivatives for lectin could not be made from erythrocyte agglutination studies due to the poor solubility of *p*-nitrophenyl galactoside in PBS. However the α-isomer was found to be 20 times more inhibitory when soluble guar gum was used in place of erythrocytes (data not shown). This difference also explained the high affinity of the lectin towards guar gum and its inertness towards Sepharose. This marked preference for the α-anomer over the β-anomer is not observed in other predominantly α-galactoside binding lectins. Thus in the case of *Bandeirae simplicifolia* I lectin (Hayes and Goldstein, 1974) and pea nut (*Arachis hypogaea*) lectin (Lotan *et al.*, 1975), the α-galactoside moieties are only 1.3 and 1.5 times better inhibitors than the β-anomers, respectively.

The lectin did not show specificity towards any blood group in hemagglutination reactions. The erythrocytes from all the blood groups were agglutinated with equal efficiency. In order to compare the avidity of various sugars towards the lectin, their capacities to inhibit hemagglutination on prior incubation with lectin was studied. Making two-fold serial dilutions, the minimum concentration of each sugar that inhibits twice the minimum hemagglutinating amount of lectin was determined (table 1). N-acetyl D-galactosamine, though 4 times better compared

Table 1. Inhibition capacity of common sugars on agglutination of erythrocytes by jack fruit seed agglutinin.

Sugar	Minimum concentration (mM) required to inhibit twice the hemagglutinating amount of lectin.
Glucose	N.I.[a]
Galactose	200
Mannose	N.I.[a]
Methyl-α-D-glucoside	N.I.[a]
Methyl-α-D-galactoside	6
Methyl-α-D-Mannoside	N.I.[a]
p-Nitrophenyl-α-D-galactoside	0.75
p-Nitrophenyl-β-D-galactoside	N.I.[b]
D-Galactosamine	400
N-Acetyl D-galactosamine	50

Aliquots of 0.2 ml PBS containing 150 µg lectin (double the minimum amount for hemaglutination) with or without serial dilution of the sugars were incubated for an hour at 5°C and 0.05 ml of a 5% suspension of erythrocytes in PBS was added. The mixture was kept at 25°C with occasional shaking and agglutination noted after 2 h.

[a] No inhibition upto 400 mM concentration.   [b] No inhibition at the limit of solubility.

to galactose, was a very poor inhibitor, compared to *p*-nitrophenyl-α-D-galactoside or methyl-α-D-galactoside.

The molecular weight determination of native lectin by gel filtration on Biogel P-100 gave a value of 39,500 daltons. However, SDS-polyacrylamide gel electrophoresis with or without 2-mercaptoethanol showed a single band corresponding to a molecular weight of 10,500 (figure 3). It is reasonable to conclude that the lectin is a tetramer, in which 10,500 dalton polypeptides are non-covalently linked. This structural phenomenon is similar to that of concanavalin A. The present lectin showed no metal requirement for its sugar binding activity.

The homogeneous lectin after guar gum affinity column elution was passed through a concanavalin A Sepharose column and the column was thoroughly washed with PBS and then eluted with the same buffer containing 500 mM α-methyl glucoside. The hemagglutinating activity was associated with the protein fraction eluted with α-methyl glucoside, indicating that the lectin is a glycoprotein. Estimation by the phenol-sulphuric acid method with glucose as standard showed 3% neutral sugar content in the lectin.

Acknowledgements

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In vivo effect of L-ascorbic acid on benzo(α)pyrene metabolite-DNA adduct formation in rat liver

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Abstract. Pretreatment of male Wistar rats with L-ascorbic acid results in a decrease in the *in vivo* covalent binding of benzo(a)pyrene to hepatic nuclear DNA. *In vitro* formation of this adduct is also found to be low in liver slices and in liver nuclei of pretreated rats. No inhibition of the adduct formation is, however, observed when benzo(a)pyrene and exogenous DNA are incubated with liver microsomes isolated from ascorbic acid treated rats. It appears that the presence of ascorbate in the cellular or subcellular environment is essential for its inhibitory action.

Keywords. L-ascorbic acid, benzo(a)pyrene, DNA, adduct formation, carcinogenesis.

Introduction

The environmental carcinogen benzo(a)pyrene, a polycyclic aromatic hydrocarbon, requires metabolic activation by microsomal enzymes to exert its toxic, mutagenic and carcinogenic action (Gelboin *et al.,* 1972; Sims and Grover, 1974). When activated thus, the compound undergoes covalent interaction with cellular DNA (Gelboin, 1969). The formation of an adduct of DNA with the activated metabolite of benzo(a)pyrene is regarded as a critical step in the process of carcinogenesis (Sims and Grover, 1974; Jerina and Daly, 1974). We have recently shown that such an adduct formation *in vitro* as well as metabolic activation of benzo(a)pyrene by isolated microsomes are partially inhibited in the presence of L-ascorbic acid (Shah and Bhattacharya, 1980). Since ascorbic acid is known to afford protection against experimental carcinogenesis induced by chemicals (Schlegal *et al.,* 1970; Shamberger, 1972; Slaga and Bracken, 1977) including that by benzo(a)pyrene (Kallistratos and Fasske, 1980), it is desirable to study the effect of administration of ascorbic acid on the formation of the carcinogen-DNA adduct in the liver of rats. We report here results of our investigation with benzo(a)pyrene.

Materials and methods

Benzo(a)pyrene was obtained from Calbiochem, La Jolla, California, USA. [^{3}H]-Benzo(a)pyrene (Sp. act. 3.7 Ci/m mol) was a product of Isotope Division, Bhabha

Atomic Research Centre, Bombay. [³H]-Benzo(a)pyrene was diluted suitabl
non-radioactive material prior to use. L-Ascorbic acid was from E.M
Darmstad, W. Germany and calf thymus DNA was from Sigma Chemical C
Louis, Missouri, USA.

Male Wistar rats (100 – 150 g) were kept in standard cages and fed a labor
stock diet and water *ad lib*. Treatment consisted of a single intraperitonial inje
of L-ascorbic acid (100 or 300 mg/kg body weight). For *in vivo* experim
further treatment involved intraperitonial administration of 20 mg/kg
benzo(a)pyrene (64.2 mCi/m mol) 2 h after ascorbic acid injection. 16 h
administration of [³H]-benzo(a)pyrene the animals were sacrificed by decapit
and the livers were quickly removed, washed free of blood and used for isolati
nuclear DNA. For *in vitro* experiments, the livers were removed from rats 2 h
ascorbic acid treatment and used for the preparation of slices, nuclei
microsomes. All subsequent operations were carried out at 4°C unless othe
mentioned.

Liver slices were prepared by cutting sections of 0.3-0.4 mm thickness us
Stadie-Riggs tissue slicer. The slices were suspended in Krebs-Ringer solu
phosphate buffer, pH 7.4. The nuclear fraction was purified from the liv
incorporating certain modifications in the method employed by Farber
(1974). Whole liver or liver slices were homogenized in 3 volumes of 0.
sucrose-3 mM MgCl₂ using a glass teflon homogenizer. The homogenat
centrifuged at 800 *g* for 10 min. The supernatant was saved for the isolati
microsomes. The nuclear pellet was suspended in 1.7 M sucrose and layered
cushion of 2.1 M sucrose. Intact and pure nuclei were obtained by centrifugat
42,500 *g* for 1 h. The purified nuclear fraction was suspended in 0.25 M sucr
mM MgCl₂ at a concentration of 8 g liver equivalent/ml. The supernatan
centrifuged at 24,000 *g* for 10 min to remove mitochondria, and the resu
supernatant from this step was further centrifuged at 110,000 *g* for 1.5 h to o
pure microsomal fraction. This was suspended in 0.25 M sucrose-1 mM MgC
give a concentration of 0.5 g liver equivalent/ml. Liver slices, nuclei
microsomes were incubated independently with [³H]-benzo(a)pyrene u
appropriate conditions. Exogenous DNA (calf thymus) was added in the ca
microsomes.

The isolation of nuclear DNA was performed according to the follo
procedure. The nuclear fraction was washed with 0.14 M NaCl-0.02 M EDTA
the nuclei were reisolated by centrifugation at 800 *g* for 10 min. The final nu
pellet was lysed in 4 volumes of 1 M NaCl-0.1 M EDTA-2% Na-dodecyl sulp
60°C for 10 min. After cooling in ice the sample was extracted with an equal vol
of chloroform: isoamyl alcohol: phenol (24:1:25) followed by two fu
extractions with chloroform : isoamyl alcohol (24:1). The DNA was precipi
from the aqueous layer by 2.5 volumes of cold ethanol, washed in ethanol
dissolved in 0.14 M NaCl-0.014 M Na-citrate, pH 7.0. Ribonuclease (200
previously heated at 85°C for 10 min, was added and the mixture was incubat
37°C for 15 min. The mixture was cooled and extracted once with water satu
phenol : *m* cresol (4:1) and twice with chloroform : isoamyl alcohol (24:1)

DNA was finally precipitated from the aqueous phase with 2 volume of cold ethanol, washed and dissolved in 0.14 M NaCl–0.014 M Na-citrate, pH 7.0. An aliquot was used to measure $A_{260 nm}$, and a second aliquot containing about 2 mg DNA was hydrolysed with an equal volume of 1 M perchloric acid for 15 min at 70°C. The sample was cooled, neutralized with 1 M NaOH and centrifuged. The hydrolysed sample was counted for radioactivity in a Beckman model LS-100 liquid scintillation spectrometer using a Triton X-100-toluene cocktail (7 g PPO, 200 mg POPOP, 350 ml Triton X-100 and 650 ml toluene). The DNA from the microsomal mixture was isolated, purified and the bound carcinogen measured according to a procedure reported earlier (Shah and Bhattacharya, 1980).

Results

The result of the *in vivo* experiment is shown in figure 1. Subsequent to administration of [³H]-benzo(a)pyrene, the DNA bound carcinogen was measured to be 1.89 μmol/mol DNA-P in the hepatic nuclei of normal rats. Pretreatment of rats with L-ascorbic acid reduced the *in vivo* binding of the carcinogen. The respective inhibition of adduct formation was 60% at 100 mg/kg^{-1} and 76% at 300 mg/kg body weight dose levels. This inhibition by ascorbic acid is considered highly significant, inspite of the fact that the extent of binding was low.
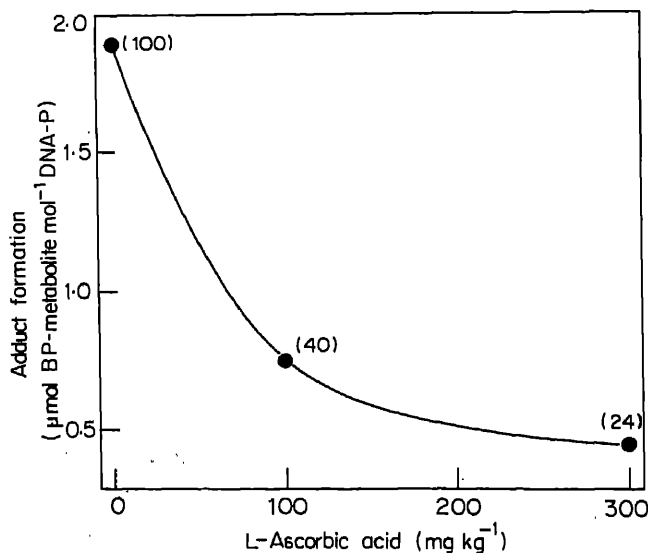
Figure 1. *In vivo* adduct formation in hepatic nuclei of rats pretreated with L-ascorbic acid.

Adduct formation was calculated from the amount of radioactive benzo(a)pyrene-metabolite covalently bound to DNA (millimolar extinction coefficient of DNA-P at 260 nm was taken as 6.6). Each value represents average from 4 rats. Figures in parenthesis are per cent of control value.

The extent of *in vitro* binding of [³H]-benzo(a)pyrene to DNA in three different liver preprations obtained from ascorbic acid treated rats is presented in table 1.

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Table 1. *In vitro* adduct formation in liver subcellular preparations of rats pretreated with L-ascorbic acid.

L-Ascorbic acid treatment	Adduct formation, µmol benzo(a)pyrene-metabolite mol^{-1}		
	Slices	Nuclei	Microsome
None (control)	2.80±0.15 (100)	2.32±0.20 (100)	28.12±3.1 (100)
100 mg/kg	2.31±0.20 (82)	2.30±0.24 (99)	34.92±4.5 (124)
300 mg/kg	1.67±0.22 (60)	1.50±0.25 (64)	26.52±3.9 (94)

Liver slices, nuclei and microsome were prepared from normal and ascorbic acid treated rats as described in the text. *In vitro* reaction conditions were as follows:

Liver slices — 5 g fresh weight of slices were suspended in 15 ml Krebs-Ringer solution — phosphate buffer, pH 7.4 and incubated at 37°C with 0.4 µmol [³H]-benzo(a)pyrene (322.5 mCi/m mol) for 2 h with constant shaking. Nuclear fraction was then prepared.

Liver nuclei — each reaction mixture (2 ml) contained Na-phosphate buffer, pH 7.4, 30 µmol, EDTA, 50 µmol, NADPH, 1 mg; [³H]-benzo(a)pyrene, 0.2 µmol (322.5 mCi/m mol), control microsome, equivalent of 100 mg fresh liver (about 1 mg protein) and nuclei, equivalent of 2 g fresh liver. Incubation was at 37°C for 30 min. Nuclear DNA was isolated and purified as described in the text.

Liver microsome — reaction condition was same as in nuclei except that sp. act. of [³H]-benzo(a)pyrene was 129 mCi/m.mol and nuclei were omitted, but calf thymus DNA (1 mg) was added. DNA was reisolated and purified according to earlier method (Shah and Bhattacharya, 1980). Adduct formation was calculated from the amount of covalently bound radioactive metabolite to DNA (millimolar extinction coefficient of DNA-P at 260 nm was 6.6). Each value represents average from 4 rats with standard deviation, and figures in parenthesis are per cent of control of a respective group.

When [³H]-benzo(a)pyrene was incubated with liver slice preparations from ascorbic acid treated rats, covalent binding to nuclear DNA observed to be low as compared to the binding observed in liver slices of normal animals. The degree of inhibition was somewhat dose related, being 18% and 40% with 100 mg/kg and 300 mg/kg body weight respectively. With liver nuclei, inhibition in adduct formation was observed only in preparations from rats treated with ascorbic acid at 300 mg/kg. The magnitude of ascorbic acid inhibition was, therefore, more pronounced in liver slice preparations than in isolated nuclei.

The binding of [³H]-benzo(a)pyrene to exogenous DNA mediated by isolated microsome was found to be markedly higher. This was mainly due to more efficient utilization of substrate by the microsomal enzyme and easy accessibility of the metabolite to native DNA which was exogenously added. This was in contrast to the *in situ* situation in liver slices and nuclei where the metabolite had limited access to DNA in regions of chromatin (Jahn and Litman, 1979). The microsomal activity to bring about the binding of benzo(a)pyrene metabolite to exogenous DNA, however, was not found to be inhibited following ascorbic acid treatment of rats. The slightly higher activity observed at 100 mg/kg body weight level was perhaps non-specific with respect to ascorbic acid, in as much as no further stimulation was noted with a higher dose of ascorbic acid.

Discussion

Liver microsomal enzymes metabolise benzo(a)pyrene subsequent to its administration to rats. It has been established that benzo(a)pyrene-7,8-dihydrodiol, a metabolite among others which are produced, is more potent as a carcinogen than benzo(a)pyrene (Kapitulnik *et al.*, 1977). When activated by microsomal preparation, this product also binds to DNA (Borgen *et al.*, 1974). The reactive metabolite involved in the binding to DNA has been identified as a diol epoxide of benzo(a)pyrene (Sims *et al.*, 1974). One stereoisomer (±)-7β, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, termed benzo(a)pyrene-diol epoxide I, is the predominant species generated enzymatically from benzo(a)pyrene-7,8-dihydrodiol (Huberman *et al.*, 1976; Yang *et al.*, 1976), and is the ultimate carcinogenic metabolite of benzo(a)pyrene. The benzo(a)pyrene-diol epoxide I is also carcinogenically most potent (Kapitulnik *et al.*, 1977). In the present study we have measured the formation of the adduct DNA-benzo(a)pyrene diol epoxide I in rat liver using [^{3}H]-benzo(a)pyrene. We have also demonstrated that in the ascorbic acid treated rats this adduct formation is inhibited at the cellular and nuclear levels but not at the microsomal level. We had shown earlier that addition of L-ascorbate inhibited the adduct formation partially but significantly *in vitro* in a microsome mediated reaction using [^{3}H]-benzo(a)pyrene or [^{3}H]-benzo(a)pyrene-7,8-dihydrodiol, and in a non-enzymatic reaction between [^{3}H]-benzo(a)pyrene diol epoxide I and DNA (Shah and Bhattacharya, 1980). These studies utilized microsome from normal rats and exogenous DNA.

The absence of any inhibition of microsomal activity while there is inhibition at the whole liver cell level due to *in vivo* ascorbic acid treatment indicates that L-ascorbate does not have any action at the organelle level. At the cellular level, it is predominantly the microsomal enzymes which convert benzo(a)pyrene to benzo(a)pyrene-diol epoxide I, but ascorbic acid treatment does not seem to inhibit the enzymatic functions of the microsome. It appears, therefore, that the presence of ascorbate in the cellular environment is essential to bring about an inhibitory response. Purified microsomal fractions are not expected to contain any ascorbate, while the whole cells (in slices) and nuclear fractions can have a substantial amount of ascorbate, especially after administration of high doses (Burch, 1961).

Regarding the different inhibitory responses to ascorbate under *in vivo* and *in vitro* binding conditions two points are to be noted. First, at 100 mg/kg dose level ascorbate inhibits adduct formation in liver to the extent of 60% *in vivo* but 18% *in vitro*. Thus the inhibition is far more efficient *in vivo*. This is probably because of ready replenishment of ascorbate in liver through the blood and lymphatic systems assuring its continued presence. Ascorbate is also known to induce fragmentation in DNA (Yamafuji *et al.*, 1971; Stitch *et al.*, 1977) which may result in possible repair synthesis. Sixteen hours after benzo(a)pyrene treatment bound benzo(a)pyrene will thus be considerably reduced. Secondly, on increasing the ascorbate dose from 100 mg/kg to 300 mg/kg, the inhibition of *in vivo* adduct formation increases from 60% to only 76%, while inhibition under *in vitro* binding

conditions increases from 18% to 40%. Thus the *in vitro* binding system responds better to increase in the dose of ascorbate. Since these livers are obtained only 2 h after ascorbic acid treatment, the marked inhibition is probably due to the increased concentration of ascorbate present in liver slices.

Under *in vitro* binding conditions with the nuclear preparation, the inhibition of adduct formation is not apparent at 100 mg/kg body weight dose level, while at 300 mg/kg dose, it is as much as that in liver slices. Liver nuclei from rats treated with a low level of ascorbate may not have enough ascorbate to bring about reduction in the availability of benzo(a)pyrene-diol epoxide I generated at the outer nuclear envelope and by exogenously added microsome for its subsequent reaction with DNA. In the case of liver slices, on the other hand, effective inhibition is brought about by the presence of ascorbate both in cytosol and in the nuclei. Significant inhibition in nuclear preparation at 300 mg kg^{-1} dose level, therefore, seems to be a reflection of adequate ascorbate in the nuclei. It is evident furthermore that there is a limit to the inhibitory action of ascorbate in as much as the inhibition on adduct formation is neither linear with dose both *in vivo* and *in vitro*, nor complete as shown in the present study and in earlier studies (Shah and Bhattacharya, 1980).

The mode of action of ascorbic acid in inhibiting the formation of DNA-benzo(a)pyrene diol epoxide I complex is not clearly understood. One explanation is that ascorbic acid, owing to its existence as ascorbate anion at physiological pH (Edgar, 1974), can be considered as a nucleophile which can react with the electrophilic benzo(a)pyrene diol epoxide I. We presume that the *in vivo* effect of L-ascorbic acid with regard to carcinogenesis induced by benzo(a)pyrene or other chemicals can be explained on this basis. Further investigation is clearly needed to verify this hypothesis.

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Effect of restraint stress on rat brain serotonin

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Abstract. Restraint-induced stress in rats was found to enhance steady state concentrations of whole brain and hypothalamic serotonin, at 1, 2 and 4 h after immobilization. The increase was maximal at 1 h and tended to decline thereafter. The rate of accumulation of rat brain serotonin, in pargyline pretreated animals, was significantly enhanced after restraint stress. Bilateral adrenalectomy and metyrapone, an endogenous corticoid synthesis inhibitor, failed to affect restraint stress (1 h)-induced increase in rat brain serotonin levels. Thus restraint stress-induced autoanalgesia and potentiation of the pharmacological actions of several centrally acting drugs, in rats, are serotonin-mediated responses. The results also indicate that restraint stress-induced effects on rat brain serotonin are not dependent on endogenous corticoid activity.

Keywords. Restraint stress; serotonin; adrenalectomy; metyrapone.

Introduction

Serotonin has been reported to be essential for maintaining physiological and psychological homeostasis, during exposure of animals to stressors (Saito, 1976). However, the effects of different stress situations, including immobilization, on brain serotonin are equivocal (Selye, 1976). Thus, restraint stress has been reported to enhance (Rosecrans and De Feo, 1965; Culman *et al.,* 1978), have no effect (Curzon *et al.,* 1972) or decrease (Curzon and Green, 1969, 1971) rat brain serotonin concentrations.

In recent communications from this laboratory, restraint stress-induced autoanalgesia (Bhattacharya *et al.,* 1978), and hypothermia (Amar and Sanyal, 1981) were shown to be serotonin-mediated responses. Furthermore, restraint stress-induced potentiation of morphine analgesia, anticonvulsant actions of phenobarbitone and diphenylhydantoin, hexobarbitone hypnosis and haloperidol catalepsy, in rats, were reported to be serotonin-mediated effects, since they were attenuated after pretreatment with pharmacological agents known to inhibit central serotonergic activity (Bhattacharya and Bhattacharya, 1979, 1982; Bhattacharya, 1980). In view of these pharmacological data, it was considered worthwhile to investigate the effect of restraint stress on rat brain serotonin concentrations and turnover using the same strain of rats, environmental conditions and method of restraint as used in earlier studies. The above factors are known to affect brain serotonin concentrations and may be held responsible,

atleast partly, for the equivocal data on stress-induced changes in the levels of this neurotransmitter published from different laboratories (Green and Grahame-Smith, 1975). The effect of metyrapone, an inhibitor of endogenous corticoid synthesis (Temple and Liddle, 1970), and bilateral adrenalectomy on rat brain serotonin changes induced by restraint stress, was also investigated because of the reports indicating serotonin-mediated regulation of stress induced activation of the hypothalamo-hypophyseal-adrenal system (Vermes *et al.*, 1972, 1973) and adrenocortical regulation of brain serotonin (Scapagnini *et al.*, 1969).

Materials and methods

Male Wistar, litter-mate, albino rats (100-150 g) were used. The rats were maintained on standard diet (Hindustan Lever pellet) and housed in an air cooled room (25°C) in colony cages. Animals were fasted overnight but water was allowed *ad lib* before experimentation. Experiments were always conducted between 09.00 h and 14.00 h. The rats were immobilised in adjustable metallic restraint chambers after tying fore and hind limbs, separately and then together (Singh, 1971). Groups of rats were immobilised for 1, 2 and 4 h, removed from the restraint chamber and killed by decapitation. For estimation of hypothalamic serotonin concentrations, the hypothalamus, the region lying between the rostral borders of the optic chiasma and mammillary bodies, and medial to the area lateralis, was dissected out as quickly as possible and the hypothalami of three rats were pooled. Brain serotonin was estimated by the fluorometric method of Snyder *et al.* (1965). The rate of accumulation of rat brain serotonin, after administration of pargyline hydrochloride (75 mg/kg, i.p.), was estimated by the method of Neff and Tozer (1968). Pargyline was administered just prior to immobilisation and rats were sacrificed at 0, 1, 2 and 4 h and the rate of accumulation (μg/g/h) calculated.

In a separate set of experiments, metyrapone (20 mg/kg, i.p.) was administered twice at four hourly intervals before immobilisation. In another group of rats, bilateral adrenalectomy was performed under ether anaesthesia, a day prior to restraint. In both these groups restraint was restricted to 1 h because the peak effect of restraint stress on brain serotonin levels was achieved at this time period. Furthermore, there was a high incidence of mortality when immobilisation was extended for more than 1 h in adrenalectomized rats. Adequate controls, including sham-operated rats, were maintained in all experiments.

Statistical evaluation was done by the Student's *t* test.

Results

Restraint stress for 1, 2 and 4 h, significantly enhanced whole brain and hypothalamic serotonin concentrations, the increase being maximal at 1 h in both tissues. Thereafter, there was a steady decline, though the serotonin concentrations of whole brain and hypothalamus at 4 h were significantly higher than control levels. Though hypothalamic serotonin concentrations were higher than that of whole brain, there was no qualitative difference in the stress-induced change in this parameter (figure 1).
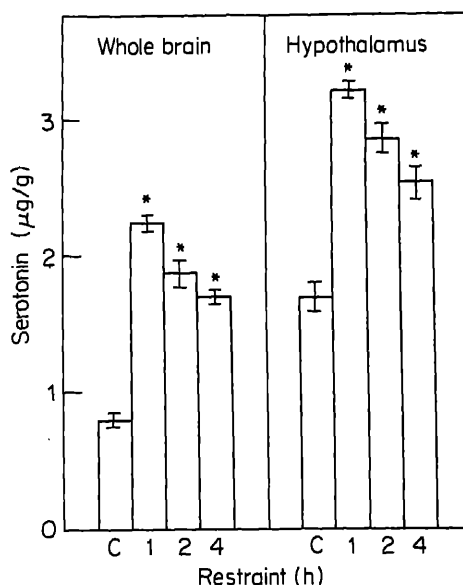
Figure 1. Effect of restraint stress (1, 2 and 4 h) on rat whole brain and hypothalamic serotonin concentrations, controls (C) were unrestrained rats. Each bar represents Mean ±S.E. of six experiments. Three hypothalami have been pooled for one estimation. * Represents P < 0.001 in comparison to control.

Metyrapone, sham adrenalectomy and bilateral adrenalectomy had no effect on rat brain serotonin as compared to untreated controls, nor did they affect restraint stress-induced increase in serotonin concentration (at 1 h) (table 1).

Table 1. Effect of bilateral adrenalectomy and metyrapone on restraint stress (1 h) — induced increase in rat brain serotonin concentrations.

Treatment	Serotonin (µg/g wet tissue)	
	Control	Restrained
None	0.82 ± 0.02 (6)	2.24 ± 0.03 (6)
Sham adrenalectomy	0.91 ± 0.06 (5)	2.48 ± 0.1 (5)
Adrenalectomy	0.99 ± 0.08 (5)	2.82 ± 0.12 (5)
Metyrapone	0.78 ± 0.03 (6)	2.28 ± 0.05 (6)

Values represent Mean ±S.E. Figures in parenthesis indicate number of animals in each group.

The rate of accumulation of rat brain serotonin was enhanced by restraint stress, in pargyline-pretreated animals. The mean rate of serotonin accumulation (µg/g/h) in pargyline-treated and stressed-pargyline-treated rats was 0.76 and 1.25 respectively. The increase in the rate of accumulation (µg/g/h) in stressed rats was thus 0.49 (figure 2).
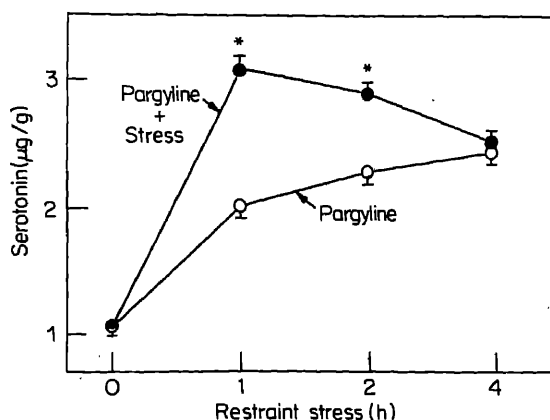
Figure 2. Effect of restraint stress (1, 2 and 4 h) on the rate of accumulation of rat brain serotonin in pargyline hydrochloride (75 mg/kg, i.p.) pretreated rats. Each point represents Mean ±S.E. of six experiments. Note the significant (* P<0.001) increase in rate of accumulation at 1 and 2 h of restraint in comparison to control.

Discussion

A number of manipulations have been used in the laboratory which have some degree of analogy to physiological stress. Forced immobilisation is one of the best explored models of stress in rats. This model combines emotional stress (escape reaction) and physical stress (muscle work), resulting in both restricted mobility and aggression. Furthermore, painful stimuli are not directly involved in restraint stress and hence this form of stress is probably more akin to physiological stress (Selye, 1976).

Restraint stress was found to enhance serotonin concentrations in both whole brain and hypothalamus with maximal increase found at 1 h after immobilization. Earlier studies on restraint stress and brain serotonin in rats have reported an increase (De Schaepdryver *et al.*, 1969; Culman *et al.*, 1978), no effect (Curzon *et al.*, 1972) or a decrease (Curzon and Green, 1969, 1971) in rat brain serotonin concentration following immobilisation. Repeated restraint has also been shown to enhance rat brain serotonin concentrations, indicating absence of tolerance (Rosecrans and De Feo, 1965). These apparently discrepant data may be due to the different strains of rats used, difference in environmental conditions, including ambient temperature, and difference in the methodology of restraint, all of which are known to affect serotonin concentrations (Green and Grahame-Smith, 1975). The paradox becomes even more evident when it is noted that discrepant results on restraint stress-induced changes on rat brain serotonin have been reported from the same laboratory (Curzon and Green, 1969, 1971; Curzon *et al.*, 1972). Restraint stress-induced increase in hypothalamic serotonin levels observed in the present study are in confirmity with the observations of Mueller *et al.* (1976), who have reported significant increase of hypothalamic serotonin concentrations at 45 and 150 min of restraint stress. However, the data of Telegdy and Vermes (1976) contradict the present observations and that of Mueller *et al.* (1976).

Restraint stress was found to enhance the rate of accumulation of rat brain serotonin in pargyline treated animals. The rate of accumulation of serotonin after inhibition of the major metabolic pathway by a monoamine oxidase inhibitor like pargyline, indicates the rate of synthesis of the amine which, in turn, gives an assessment of the rate of its turnover (Neff and Tozer, 1968). Our data are in conformity with that of some earlier reports (Nistico and Preziosi, 1969; Ladisich, 1974). Immobilisation has been reported to selectively enhance rat brain tryptophan levels (Knott *et al.,* 1973), thus making available the precursor amino acid for serotonin synthesis.

Elevation of adrenocortical secretion by stress has been reported to influence brain serotonergic activity, and hypothalamic serotonin is thought to have a role in stress-induced activation of the hypothalamo-hypophyseal-adrenal system (Vermes *et al.,* 1972, 1973). Enhanced adrenocortical activity or treatment with cortisol, corticosterone and synthetic glucocorticoids have been reported to decrease brain serotonin levels, probably by inducing hepatic tryptophan pyrrolase activity, resulting in diversion of tryptophan from the pathway of brain serotonin synthesis (Curzon and Green, 1968; Scapagnini *et al.,* 1969). In keeping with these observations bilateral adrenalectomy has been reported to enhance rat brain serotonin levels (De Schaepdryver *et al.,* 1969). However, adrenalectomy has also been reported to inhibit trytophan hydroxylase activity, an effect reversed by corticosterone (Rastogi and Singhal, 1978). In the present study, bilateral adrenalectomy had no effect on rat brain serotonin, nor did the surgical manoeuvre affect restraint stress-induced increase of the amine levels. However, it should be pointed out that the latter effect was investigated only at 1 h restraint because mortality during extended immobilisation was high in these animals. Metyrapone, which inhibits the synthesis of corticosterone, the major endogenous corticoid in rats, by decreasing 11-β-hydroxylation (Temple and Liddle, 1970) failed to affect rat brain serotonin levels in unrestrained and restrained animals. These data suggest that peripheral corticoid activity is not related to stress-induced changes in brain serotonin concentrations. Curzon (1971) has inferred that many factors are likely to play a role in stress-induced effects on central serotonergic activity, and it cannot be said that the brain serotonin metabolism is entirely at the mercy of the adrenal gland.

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A new repressible alkaline phosphatase in *Neurospora crassa* EM 5297a

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Abstract. *Neurospora crassa* Em 5297a can utilize sodium β-glycerophosphate as a sole phosphorous source (in the place of KH_2PO_4). Under these conditions a repressible alkaline phosphatase is elaborated which has different pH optimum towards β-glycerophosphate (10.2) and pyrophosphate (9.0) as substrates. This enzyme does not require any metal ion for its activity and could be assayed in the presence of EDTA. However, under conditions of cobalt toxicity, the activity of this enzyme is high and is decreased in copper and nickel toxicities.

Keywords. *Neurospora crassa* Em 5297a; repressible alkaline phosphatase; β-glycerophosphate.

Introduction

Recent studies have revealed the presence of inorganic phosphate repressible alkaline and acid phosphatases in *Neurospora crassa* (Nyc *et al.*, 1966; Kader *et al.*, 1968; Nyc, 1967; Jacobs *et al.*, 1971). Unlike the constitutive enzyme, the repressible alkaline phosphatase of *N. crassa* 1A has no metal requirement and is distinguished from the former by the fact that it is assayed in the presence of EDTA (Nyc *et al.*, 1966). In contrast to the above, we found in *N. crassa* Em 5297a a repressible alkaline phosphatase elaborated when this strain was grown on β-glycerophosphate as a phosphorous source. This phosphatase differs in certain of its properties from the constitutive alkaline phosphatase of Em 5297a (Ku and Blumenthal, 1961) as well as the repressible enzyme of *N. crassa* 1A (Nyc *et al.*, 1966). Some features of this new enzyme are reported in this paper.

Materials and methods

Chemicals

All chemicals including sodium β-glycerophosphate and pyrophosphate were of Analytical grade and were obtained from British Drug House, India.

Organism and growth conditions

A wild strain, *Neurospora crassa* Em 5297a used in the present studies was maintained by weekly subcultures on agar slants. It was grown for 72 h at $30 \pm 1°C$ in 50 ml conical flasks containing 10 ml basal medium. The medium composition was as described earlier except that it contained 0.04% disodium β-glycero-

phosphate monohydrate and 0.015% KCl in the place of KH_2PO_4 (Venkateswerlu and Sivarama Sastry, 1973). To study the effect of metal toxicities on the elaboration of the repressible phosphatase, Co^{2+}, Ni^{2+} and Cu^{2+} were also included at 600, 300 and 800 µg/10 ml respectively in the culture medium. The pH of the medium was always adjusted to 4.5-5.0.

The mycelia (72 h old) were washed with ice-cold distilled water, pressed dry between the folds of filter paper and ground at 0-4°C with twice their weight of glass powder in a mortar and pestle with 0.5% sodium deoxycholate, pH 7.0 (5ml/150 mg fresh mycelial weight). The homogenate was centrifuged at 4°C for 15 min at 10000 g and the supernatant collected. The residue was reextracted likewise with another 2-3 ml of deoxycholate. The pooled extracts constituted the enzyme source. The protein content of the extract was determined by the method of Lowry *et al.* (1951).

Enzyme assay

Enzyme activity was assayed according to the procedure described by Nyc *et al.* (1966). The incubation mixture contained 10 µmol of sodium β-glycerophosphate (or sodium pyrophosphate as needed), 1 µmol EDTA, 0.1 ml of 0.2 M glycine-NaOH buffer, pH 10.2 (or pH 9.0 when sodium pyrophosphate was the substrate) and a suitable aliquot of enzyme extract in a total volume of 0.9 ml. Incubation was for 20 min at 37°C and the reaction was terminated by adding 0.1 ml of 50% trichloroacetic acid. The tubes were centrifuged and the liberated inorganic phosphate was determined in the supernatant by the method of Fiske and Subba Rao (1925). The specific activity of the enzyme is expressed as µmol of P_i liberated per mg of protein in 20 min.

The constitutive alkaline phosphatase of *N. crassa* is not assayable in the presence of EDTA and hence this assay differentiates between the constitutive and repressible enzymes in question.

All experiments were repeated a minimum of four times, and values were invariably reproducible to within 5-10%.

Results and discussion

The presence of a repressible acid phosphatase has been reported by Grove and Marzluf (1980) in *N. crassa* cultured in the presence of ribonucleic acid as phosphate and nitrogen sources. We report repressible alkaline phosphatase in *N. crassa* Em 5297a, during growth on sodium β-glycerophosphate as a sole phosphorous source.

This enzyme was not detectable in mycelial extracts prepared from *N. crassa* cultures grown in presence of inorganic phosphate in the medium. This indicates that inorganic phosphate represses the phosphatase reported here and that it is similar to the enzyme studied by Nyc *et al.* (1966) in this respect.

The growth response of *N. crassa* Em 5297a towards β-glycerophosphate as a sole phosphorous source and the enzyme activity under these conditions are shown in figure 1. It is interesting that whereas growth is a function of
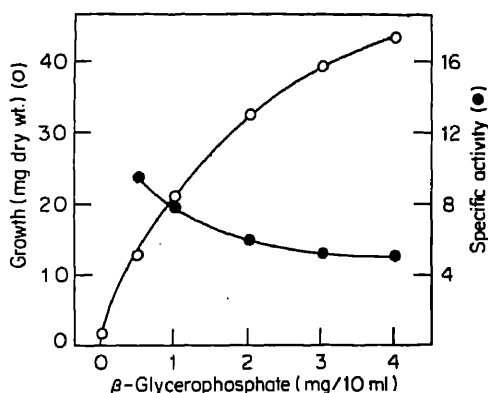
Figure 1. The growth of *N. crassa* on β-glycerophosphate and the activity of a repressible alkaline phosphatase. The mold was grown on 10 ml basal medium containing varying amounts of sodium β-glycerophosphate and 0.015% KCl for 72 h in two sets of flasks. One set of mycelia was used for determining dry weights (O) and the other for enzyme assay (●) as described in text.

glycerophosphate concentration in the range 0–4 mg/10 ml medium, activity of the repressible phosphatase is highest with low concentrations of glycerophosphate under conditions of suboptimal growth. Since the enzyme is absent during growth on KH_2PO_4 and is elaborated only on glycerophosphate as the phosphorous source, the data suggest that intracellular inorganic phosphate formed probably represses the enzyme *in vivo*.

The pH optimum for this enzyme was determined using sodium β-glycero-phosphate and sodium pyrophosphate as substrates. The results are shown in figure 2.
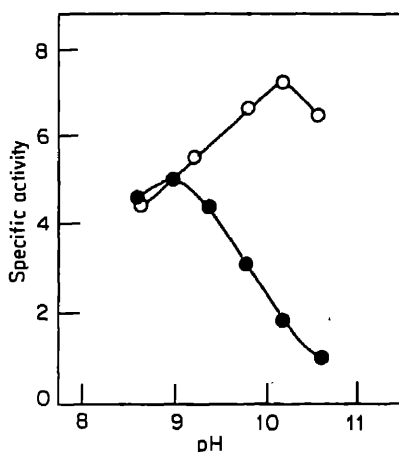
Figure 2. pH optima of repressible alkaline phosphatase from *N. crassa* Em 5297a. The enzyme preparation was obtained from *N. crassa* mycelia, grown for 72 h on 10 ml of basal medium containing 0.04% β-glycerophosphate. The enzyme assay was as described in text using either β-glycerophosphate (O) or pyrophosphate (●) as substrates.

The phosphatase from strain Em 5297a exhibits pH optima of 10.2 and 9.0 for glycerophosphate and pyrophosphate respectively, whereas the enzyme reported by Nyc *et al.* (1966) exhibited only one pH optimum for both the substrates. Further, this enzyme is 70% as active towards sodium pyrophosphate as towards sodium β-glycerophosphate (enzyme activities obtained at the respective pH optima have been compared.) In contrast, the enzyme from strain 1A has been reported to be only 28% as active towards pyrophosphate (Nyc *et al.* 1966). The K_m Value for the enzyme from strain Em 5297a is 3.68 mM with β-glycerophosphate as the substrate. The differences between the repressible alkaline phosphatases of *N. crassa* Em 5297a and strain 1A are summarized in table 1 and indicate that the two enzymes are somewhat different in their properties.

Table 1. Comparison of Properties of the repressible alkaline Phosphatases of *N. crassa* strains Em 5297a and 1A.

	Property	Em 5297a	1A*
1.	pH Optimum with		
	a) β-Glycerophosphate	10.2	9.0
	b) Pyrophosphate	9.0	9.0
2.	% Activity towards		
	a) β-Glycerophosphate	100.0	100.0
	b) Pyrophosphate	70.0	28.0
3.	Km value	3.68 mM	1.0 mM
	(For β-glycerophosphate)		

* Values taken from reference 1.

N. crassa Em 5297a was grown in presence of 0.04% β-glycerophosphate for 72 h and the enzyme was assayed using β-glycerophosphate or sodium pyrophosphate as described in text.

Although, the repressible alkaline phosphatase of *N. crassa* has no metal requirement for activity, Hochberg and Sargent (1973) reported that iron and zinc enhance the synthesis of this enzyme and that copper inhibits its synthesis. In parallel studies to examine the effect of some metal ions we found that enzyme activity in copper and nickel toxic conditions in *N. crassa* Em 5297a was only 15% and 87% of control cultures (see table 2). However, a 50% increase in the enzyme activity was observed in the case of cobalt toxic cultures. These observations indicate that some metal ions like iron, zinc and cobalt may have some role in the biosynthesis of this enzyme as suggested by Hochberg and Sargent (1973).

Table 2. Effect of metal toxicities on the new repressible alkaline phosphatase from *N. crassa* Em 5297a.

Metal	Conc. of metal ion during growth (μg/10 ml medium)	Specific activity
None	Control	7.15 (100)
CO^{2+}	600	10.20 (142.7)
Ni^{2+}	300	6.22 (87.0)
Cu^{2+}	800	1.05 (14.80)

The organism was grown on 10 ml basal medium containing the concentration of metal ions as indicated above, which resulted in 50% growth inhibition along with 0.04% β-glycerophosphate for 72 h. The enzyme was assayed as described in the text. Numbers in parentheses are values expressed as per cent of control.

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Sequential release of cellulose enzymes during germination of *Trichoderma reesei* spores

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Abstract. The pattern of release of extracellular cellulase during the germination of *Trichoderma reesei* spores has been examined. The four enzymes namely, filter paper degrading enzyme, β-1,4 endoglucanase, β-glucosidase and xylanase appear sequentially in the culture broth during germination of the spores. The order of enzyme appearance is not altered by the type of carbon source in the germination medium. Measureable quantities of filter paper degrading enzyme is detected only after the outgrowth has occurred. A possible mechanism of spore germination and induction of the enzymes by insoluble cellulose is suggested.

Keywords. *Trichoderma reesei*, cellulases; spore germination; enzyme synthesis and release.

Introduction

The degradation of crystalline cellulose to glucose involves the cooperative action of atleast three enzymes namely, β-1,4 exoglucanase (cellobiohydrolase, EC.3.2.1.–) β-1,4 endoglucanase, (EC.3.2.1.21) (Wood, 1975; Petterson, 1975, Wood and McCrae, 1978). In addition, the hydrolysis of native cellulose containing hemicellulose, also needs the cooperative action of hemicellulases (Ghose and Bisaria, 1979; Wood, 1980). A variety of fungi are known to produce these enzymes extracellularly (Mandels and Andreotti, 1978) but among these, *Trichoderma reesei* QM 6A and its mutants are the best known. In this organism, the cellulase enzymes are inducible and the best inducer known todate is the insoluble substrate namely, cellulose (Mandels, 1975). In studies on cellulase production by this organism, generally spores are used as the inoculum. There is, however, no information either with regard to the early events during spore germination and outgrowth in a medium containing cellulose or as to how this insoluble polysaccharide induces the synthesis of these enzymes, especially the exoglucanase and endoglucanase. Using a thick spore suspension as the inoculum and short sampling intervals, we have tried to determine the pattern of enzyme release and its role in the early stages of spore germination and outgrowth.

Materials and methods

Culture conditions and enzyme assays: *T. reesei* QM 6A and its mutant QM 9414 used in this study were from the Army laboratory, Natick, Massachussetts USA and

were maintained on Vogel's glucose-agar slants (Vogel, 1956). For germination studies, spores of QM 9414 from 3-5 day old slants were suspended in sterile water and mixed on a vortex mixer for 5 min. The spore suspension was used to inoculate 25 ml of synthetic medium (Reese *et al.*, 1950) containing, unless otherwise stated, 1% cellulose (Carl Schleicher and Schull, West Germany) in 150 ml conical flasks. An initial concentration of about $5\text{-}6 \times 10^7$ spores/ml of the medium was used. The flasks were incubated on a rotary shaker at 30°C (230 rpm) and duplicate flasks were withdrawn at intervals of 6 h, filtered through a gooch crucible and the clear supernatants used for enzyme assays. Filter paper degrading enzyme (Fpase), β-1,4 endoglucanase (CMCase), β-glucosidase and xylanase were assayed using Whatman No. 1 filter paper strips (50 mg), carboxymethyl cellulose (CMC, BDH grade), *p*-nitrophenyl β-D glucoside (B-PNG, Sigma Chemical Co., St. Louis, Missouri, USA) and xylan (Sigma), respectively, using standard procedures (Mandels, 1975; Herr, 1979; Linko *et al.*, 1978). Enzyme activity is expressed in international units defined as micromoles of product formed/min/ml of culture filtrate. Spore germination was monitored microscopically using a drop of the culture medium before filtration. The enzyme content of the spores was determined by sonicating spores (about $1\text{-}2 \times 10^9$ spores/ml, 10 ml) suspended in citrate buffer (0.05M, pH 4.8) for 10 min using an ultrasonic cell disintegrator (Braunsonic, 1510) with intermittant cooling. The sonicated sample was then centrifuged at 13,000 *g* for 20 min in an I.E.C. refrigerated centrifuge (4°C) and the clear supernatant was used for enzyme assays. Protein content of the spore extracts was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Fungal and bacterial spores are dormant structures and the pattern of macromolecular synthesis during germination not only represents the events that occur during the return to vegetative growth but also represents the order of gene expression during this period (Steinberg and Halvorson, 1968; Garg and Tauro, 1973). The germinating *T. reesei* spore is therefore an ideal test system to understand the sequence of gene expression and also to know the early stages of cellulose hydrolysis.

Sequence of enzyme release in cellulose medium

To determine the sequence of gene expression during germination, the order of appearance of the enzymes in the culture broth was examined (figure 1). When spores were allowed to germinate in cellulose medium, the first enzyme to be detected in the culture filtrate was β-glucosidase, which appeared between 6-12 h. The level of this enzyme thereafter remained constant till about 30-36 h when a second burst of enzyme release occurred. The two main enzymes involved in the initial stages of cellulose hydrolysis, namely filter paper degrading enzyme and endoglucanase appeared between 36-42 h and 24-36 h, respectively. Xylanase, which is presumed to have a role in the hydrolysis of native cellulose, appeared between 12 and 18 h. Thus, the release of the enzymes from the germinating spores was not simultaneous but sequential. Microscopic examination of the culture broths indicated that the spore germination and outgrowth was complete between
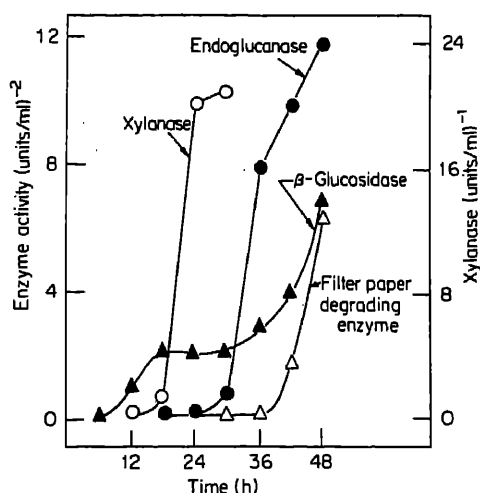
Figure 1. Sequence of enzyme release during the germination of *T. reesei* spores in cellulose medium.

24–30 h while measurable quantities of filter paper degrading enzyme appeared much later in the culture medium.

The early appearance of the β-glucosidase and the late release of endoglucanase and filter paper degrading enzyme during the germination of the spores raised questions regarding the role of this enzyme in the initial stages of cellulose hydrolysis. To examine this, two approaches were used. First, the pattern of enzyme release during the germination of spores in a medium containing glucose as the carbon source was examined. In this organism, glucose is known to repress the synthesis of β-glucosidase (Mandels, 1975). Secondly, the spores were also analyzed for their enzyme composition.

Enzyme release in glucose medium

In glucose medium, the only extracellular enzyme seen during the first 48 h was β-glucosidase, which appeared between 6-12 h as before in the cellulose medium (figure 2). However, unlike in the cellulose medium, the level of this enzyme gradually decreased and there was no second burst of enzyme release. It therefore
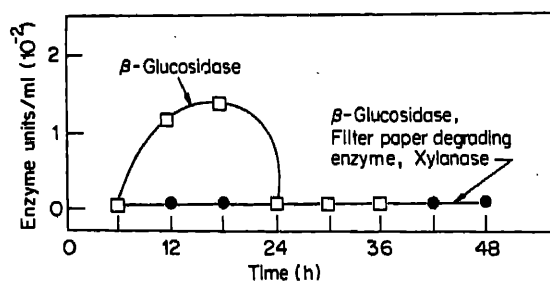
Figure 2. Release of β-glucosidase during the germination of *T. reesei* spores in glucosidase medium.

appears that this first burst of β-glucosidase corresponds to the release of the spore enzyme, while the second burst represents the release of newly synthesized enzyme.

Enzyme content of spores

Further, the enzyme composition of the dormant spores was examined (table 1). It was found that the spores contain a high level of β-glucosidase but relatively lower levels of endoglucanase and xylanase. Filter paper degrading enzyme could not be

Table 1. Enzyme composition of *T. reesei* QM 9414 spores.

Enzyme	IU/ml extract	Sp. activity*
β-Glucosidase	0.51	2.00
Endoglucanase	0.009	0.036
Filter paper degrading enzyme	Not detectable	Not detectable
Xylanase	0.01	0.04

About $1-2 \times 10^{10}$ spores in 10 ml of citrate buffer (pH 4.8, 0.05 M) were sonicated for 10 min using a ultrasonic cell disintegrator, centrifuged and the supernatant was used for enzyme and protein determination.

* IU/mg soluble protein.

detected in these extracts by the method used for its assay. From these studies, the true order of release of the new enzymes, as determined by the time of their appearance during the germination of *T. reesei* spores, appears to be xylanase, endoglucanase, β-glucosidase and filter paper degrading enzyme.

Sequence of enzyme release in phosphocellulose or xylan medium

If the order of enzyme appearance during spore germination in cellulose medium reflects the order of gene expression, then one would expect that this order should be similar and independent of the carbon source in the growth medium. To test this, the time of enzyme release during spore germination in media containing either phosphocellulose or xylan was examined (figure 3). In this organism,
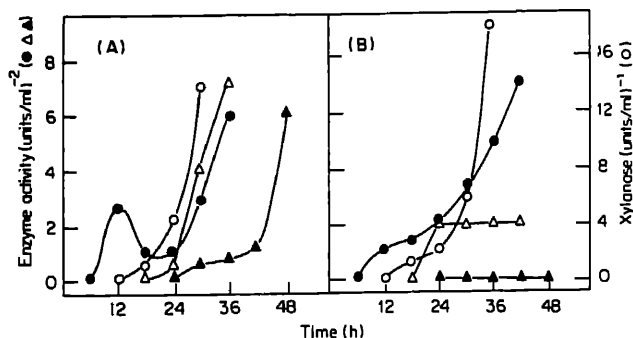
Figure 3. Pattern of enzyme release during the germination of *T. reesei* spores in phospho-cellulose (A) and in Xylan (B) media.

The germination medium contained either 1% phosphocellulose or 0.5% xylan as the carbon source. (O): Xylase, (▲): Filter paper degrading enzyme (Δ): Endoglucanase and (●): β-glucosidase.

phosphocellulose and xylan specifically elevate the levels of endoglucanase and β-glucosidase, respectively (Chaudhary, 1981). However, in media containing these substrates as carbon sources, the order of appearance of these enzymes is similar to that in cellulose medium. Thus, although the level of induction is altered, the time and order of appearance remains similar. It is therefore concluded that the order of appearance of enzyme in cellulose medium represents the true order of gene expression during the germination of the *T. reesei* spores.

Order of enzyme release in QM 6A

T. reesei QM 9414 is a mutant of strain 6A and produces a higher level of cellulase enzymes in cellulose medium (Mandels, 1975). To test whether the order of enzyme release was similar to that in the parent or whether it was altered, spores of both cultures were allowed to germinate in cellulose medium separately and the pattern of enzyme release determined as before. The sequence of enzyme release was identical in both the cultures although in mutant QM 9414, the release of these enzymes occurred slightly earlier than in the parent culture.

Discussion

Sequential synthesis of enzymes during the germination of bacterial and fungal spores has been well documented and since the germinating spores represent a synchronous system, the time of enzyme synthesis is presumed to approximate the time of gene expression (Steinberg and Halvorson, 1968). However enzyme synthesis and release are two different phenomena and it is difficult to conclude that the time of appearance of enzymes in the culture broth represents the exact time of gene expression. Although the time of appearance may not represent the exact time of synthesis, the order of appearance can be taken to represent approximately the order of gene expression during the germination of *T. reesei* spores. The four enzymes examined in this paper appear in a definite sequence irrespective of the carbon source in the growth medium.

The sequence in which the enzymes appear in the culture broth suggests that the presence of new enzymes is not required for the early stages of germination and growth. The new enzymes increase in amount only after outgrowth has occurred. This also suggests that the two spore enzymes namely the endoglucanase and β-glucosidase are adequate to generate enough soluble sugars to allow early stages of growth. Commercial cellulose contains substantial amounts of amorphous cellulose and it is likely that the small amount of spore endoglucanase released during the early stages of germination initiates its hydrolysis and it is then converted into glucose by the spore β-glucosidase. The requirement of exoglucanase enzyme is therefore necessary only when the crystalline regions of the cellulose are to be hydrolysed. These conclusions are consistent with the current predictions for cellulose hydrolysis by microbial cellulases (Wood and McCrae, 1978).

The mechanism of induction of filter paper degrading enzyme and endoglucanases by insoluble cellulose during the early stages of spore growth is at present not clear. It is likely that during the early stages of germination, the spore endoglucanase is responsible for the production of soluble cellodextrins which

then enter the germinating spores and induce both these enzymes. Alternatively, it is also likely that cellulose interacts with a specific cell surface receptor and triggers the induction of these enzymes, a mechanism similar to that described in Eucaryotic cells (Davidson and Britten, 1979). However, both these mechanisms need to be investigated.

The synthesis and release of xylanase very early during the germination process is consistent with its predicted role in native cellulose hydrolysis. Wood (1980) has reported that the extent of native cellulose hydrolysis can be increased by prior treatment with hemicellulases. Apparently, this fungus has evolved such a mechanism to enable it to first digest the hemicelluloses before it digests other regions of the native cellulose using the other enzymes.

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Studies on the properties of the variants of gamma-glutamyl transpeptidase in human urine

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Abstract. Both the high molecular weight and the low molecular weight variants of urinary γ-glutamyl transpeptidase, displayed transpeptidase (pH optimum 8.6) and autotranspeptidase (pH optimum 9.4) activities. Iodoacetamide inhibited the transpeptidase activity more efficiently than the autotranspeptidase activity with respect to both variants of γ-glutamyl transpeptidase. The high molecular weight form utilized L-glutamine as a better acceptor than L-cystine during the transpeptidation reaction whereas the reverse was the case with the low molecular weight variant. While phenylmethylsulphonyl fluoride-treated enzymes retained full activities *per se*, addition of maleic acid to the modified enzyme was found to inhibit the catalytic activities indicating a maleic acid-induced conformational change of the modified enzyme.

Keywords. Urinary γ-glutamyl transpeptidase variants; autotranspeptidation; chemical modification.

Introduction

Gamma glutamyl transpeptidase which catalyzes the transfer of glutamyl moiety from γ-glutamyl peptides and derivatives to suitable acceptors, is widely distributed in human tissues and fluids (Rosalki, 1975). The human kidney enzyme has been isolated in homogenous form and its properties have been investigated in detail (Shaw *et al.*, 1978; Tate and Ross, 1977). However, information available on the properties of urinary transpeptidase is scanty. We have recently reported the existence in the urine of two molecular forms of v-glutamyl transpeptidase, a high (> 200 000 daltons) and a low molecular weight (55000 daltons) form (Rambabu *et al.*, 1981). In this paper the properties of these two variants are reported.

Materials and methods

L-γ-Glutamyl-*p*-nitroanilide, glycylglycine, glycyl L-alanine, glycyl L-proline, glycyl L-histidine, L-leucyl L-alanine, 2,4,6-trinitrobenzene sulphonic acid (TNBS) were purchased from Sigma Chemical Company, St. Louis, Missouri,

USA. Phenylmethylsulfonyl fluoride (PMSF) was obtained from CalBiochem, Los Angeles, California, USA. 5,5´-Dithiobis 2-nitrobenzoic acid (DTNB) was the product of Pierce Chemical Company, Rockford, USA. The ultra-filtration unit (Immersible CX Starter Kit, NMWL-10000 daltons) was from Millipore Corporation, Bedord, USA. Other reagents used were of Analytical grade.

Separation of high and low molecular weight forms of γ-glutamyl transpeptidase

Urine samples obtained early in the morning from healthy individuals were processed in 50 ml batches. The sample was dialyzed at 4°C twice each time for 6 h against 100 volumes of 0.02 M Tris-HCl buffer, pH 7.5 containing 0.1 mM MgCl$_2$. The dialyzed sample was concentrated by ultrafiltration to 5 ml and chromatographed at 25°C on a Sephadex G-200 column (1.4×78 cm, bed volume 120 ml) equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 0.3 M NaCl. Elution was continued with the same buffer using a flow rate of 10 ml/h and 5 ml fractions were collected. The high molecular weight variant (fraction numbers 7, 8 and 9) and the low molecular weight variant (fraction numbers 12, 13 and 14) of the transpeptidase were concentrated by ultrafiltration and used for further studies. The enzymes were stable for 2 months on storage at 4°C.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

The transpeptidase activity was determined as described earlier (Rambabu and Pattabiraman, 1976). The routine assay system consisted of 3.2 µmol of γ-glutamyl-*p*-nitroanilide, 22 µmol of glycylglycine, 11 µmol of MgCl$_2$, 50 µmol of ammediol-HCl buffer, pH 8.6 and enzyme in a total volume of 3 ml unless mentioned otherwise. After 45 min of incubation at 37°C the reaction was stopped by the addition of 5 ml of 7.5 mM N NaOH. The liberated *p*-nitroaniline was determined by measuring the absorbance at 410 nm. The autotranspeptidase activity was also determined similarly in the absence of glycylglycine. The concentration of γ-glutamyl *p*-nitroanilide was 11.4 µmol for routine autotrans-peptidase assay. One unit of enzyme activity was defined as the amount required to release one µmol of *p*-nitroaniline under the assay conditions.

Chemical modification of amino acid residues

The amino groups of γ-glutamyl transpeptidase were modified by treatment with trinitrobenzene sulphonic acid (TNBS) (Fields, 1972). The enzyme (100 µg protein) in 3 ml was incubated with one mg of TNBS in the presence of 50 µmol of sodium borate buffer, pH 8.6 for 60 min at 28-30°C.

Sulphydryl groups were modified by treatment with DTNB (Habeeb, 1972). The protein (100 µg) was allowed to react with one mg of DTNB at 28-30°C in a volume of 3 ml for 3 h in the presence of 50 µmol of ammediol-HCl buffer, pH 8.6.

IA at different concentrations was incubated with the enzyme (100 µg protein) for 1 h in a volume of 3 ml in the presence of either 50 µmol of sodium borate buffer, pH 8.4 or 50 µmol of sodium phosphate buffer, pH 6.9 at 28-30°C (Gurd, 1972).

PMSF (2 mg) was incubated with 200 µg of the enzyme protein for 3 h at 28-30°C in a volume of 6 ml in the presence of 20 µmol of sodium phosphate buffer pH 7.5 and 0.6 ml of isopropyl alcohol (Inoue *et al.*, 1978).

The enzyme samples, after chemical modification, were dialyzed against 600 volumes of distilled water for 6 h at 4°C and the residual activities were measured. In the case of TNBS modified enzyme alone, the assay was performed in sodium borate buffer, pH 8.6 instead of ammediol-HCl buffer. With PMSF-treated enzyme, the activity was measured both in the presence and absence of different concentrations of maleic acid. In all cases suitable controls without the modifiers were used.

Paper chromatography

The enzyme (0.4 ml containing 100 µg of protein of either low molecular weight or high molecular weight variant) was incubated with 11.4 µmol of γ-glutamyl *p*-nitroanilide, 11 µmol of $MgCl_2$ and 50 µmol of ammediol-HCl buffer, pH 8.6 in a total volume of 3 ml at 37°C. At intervals of 1 h and 5 h, a 30 µl aliquot of the incubation mixture was withdrawn and subjected to descending paper chromatography on Whatman No. 1 paper for 22 h with butanol : acetic acid : water as solvent system (5 : 4 : 1). The chromatogram was dried and stained with a solution of ninhydrin (0.5% in 95% butanol) to localize the spots.

Results

Both forms of urinary γ-glutamyl transpeptidase displayed autotranspeptidase activity. The data on the rates of transpeptidase and autotranspeptidase activities as a function of pH are presented in figure 1. While the two variants of the enzyme
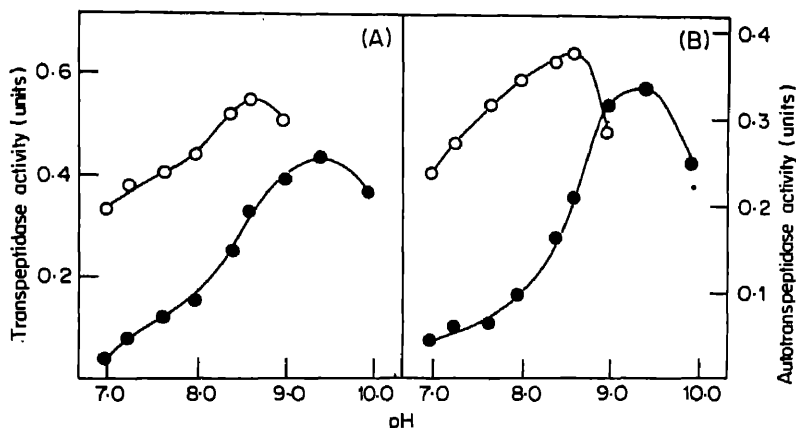
Figure 1. Effect of hydrogen ion concentration of transpeptidase (O) and autotranspeptidase (●) activities. Figure 1a represents activities with high molecular weight fraction and figure 1b represents activities with low molecular weight fraction. Buffers used are 0.05 M Tris-HCl buffer pH 7.0-8.4 and 0.05 M ammediol-HCl buffer pH 8.6-10.0.

showed an optimum pH of 8.6 for transpeptidase activity, the optimal pH for autotranspeptidase activity was around pH 9.4. The enzyme activities could not be measured beyond pH 10.0 due to rapid non-enzymic hydrolysis of the substrate. The magnitude of increase in autotranspeptidase activity was more than the transpeptidase activity in the pH range 7.0-8.6 with both the variants of urinary transpeptidase. Paper chromatographic studies (figure 2) show that urinary GGT
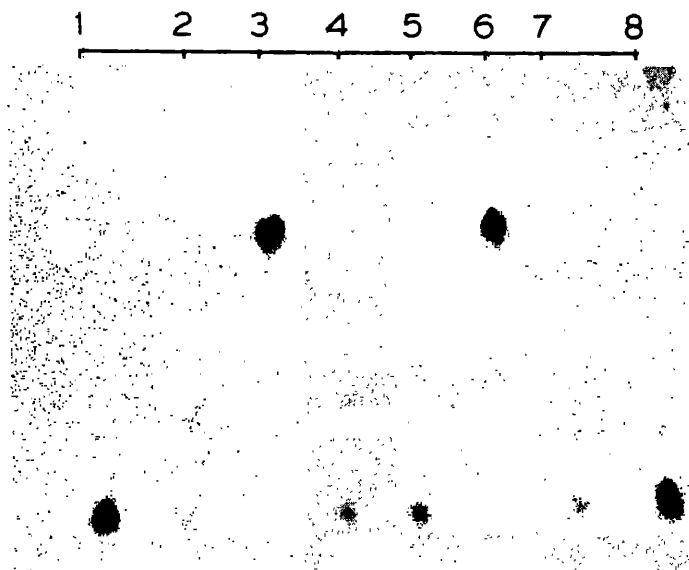
Figure 2. Descending paper chromatography of autotranspeptidase reaction products on Whatman No. 1 for 22 h (Relative mobilities were: 1 and 8-γ-glutamyl *p*-nitroanilide (36 cm); 2 and 7 — Five h incubation with high and low molecular weight GGT variants (36 cm, 29.5 cm); 2 and 6 — glutamate (11.5 cm); 4 and 5 — One h incubation with high and low molecular weight GGT variants (36 cm, 29.5 cm). Other details are given under 'Materials and methods'.

does not hydrolyse γ-glutamyl-*p*-nitroanilide to any measurable extent as evidenced by the lack of appearance of glutamic acid spots. This confirms the true autotranspeptidase action of the enzyme in the absence of the acceptor glycylglycine. The second nihydrin spot that appeared on incubation of γ-glutamyl *p*-nitroanilide with enzyme samples with an $R_{glutamate}$ value of 2.5 could be the major product of autotranspeptidation namely γ-glutamyl-*p*-nitroanilide as suggested by Orlowski and Meister (1965).

The data on the efficiency of different dipeptides and some amino acids as acceptors of γ-glutamyl moiety in the transpeptidation reaction are presented in table 1. As the bulk of the carboxy terminal residue in glycine-X acceptors increases, the transpeptidase activity was found to decrease with both forms of the transpeptidase. However, the contribution of charge differences on the substrates for the variation could not be ruled out. L-Leu-L-Ala was two thirds as efficient as Gly-L-Ala as acceptor. Among the amino acids, L-cystine and L-glutamine were

Table 1. Dipeptide and amino acids as acceptors in transpeptidase reactions with γ-glutamyl transpeptidase variants.

Acceptors	Relative transpeptidase activity	
	High mol. wt. enzyme	Low mol. wt. enzyme
Glycyl glycine	100.0	100.0
Glycyl L-alanine	76.0	78.0
Glycyl L-histidine	16.3	18.0
Glycyl L-Proline	11.5	16.0
L-Leucyl L-alanine	47.8	52.0
L-Cystine	31.7	44.5
L-Glutamine	39.0	21.6

The assay systems are the same as described under Materials and methods except that different acceptors at concentrations of 22 μmol were used in place of glycyl glycine.

found to be fairly good acceptors. While the low molecular weight variant of enzyme used L-cystine twice as efficiently as L-glutamine, the high molecular weight enzyme was more with L-glutamine than L-cystine. Most other amino acids acted as poor acceptors in the enzyme systems. The activities with L-glutamine, L-glutamic acid, L-aspargine, L-aspartic acid, L-arginine, L-lysine, L-alanine, L-leucine, L-phenylalanine, L-tyrosine, L-tryptophan, L-valine, L-serine, L-threonine, L-proline, L-hydroxyproline, L-cysteine, L-methionine varied from 0-16.2% compared to the activity with glycylglycine. Considering that autotranspeptide activity at pH 8.6 for the high molecular weight and low molecular weight forms of the enzyme were 8% and 6% respectively of transpeptidase activity, it is probable that many of them do not act as acceptors at all.

The data on the effect of chemical modifications on the two forms of urinary transpeptidase are presented in table 2. Modification of amino groups by TNBS resulted in the loss of transpeptidase as well as autotranspeptidase activities of both high molecular weight and low molecular weight forms of the enzyme. While high concentration of IA caused complete abolition of transpeptidase activity, small amounts of autotranspeptidase activities were persistent at both pH 6.9 and 8.4. DTNB treatment had no effect on the two forms of the enzyme.

PMS-treated enzyme retained full activity. However when maleic acid was included in the assay system with PMSF-treated enzymes, transpeptidase as well as autotranspeptidase activities decreased with increase in the concentration of maleic acid. Maleic acid alone had no effect on the enzymes. Maleic acid was found to reduce more of the autotranspeptidase activity of the PMSF-treated enzymes than the transpeptidase activity.

The K_m values for γ-glutamyl p-nitroanilide of the transpeptidase activity (in the presence of glycylglycine) for the high and low molecular weight variants of the transpeptidase were respectively 0.66 mM and 0.74 mM. The K_m values for γ-

Table 2. Effect of chemical modification on the activities of the two variants of the urinary transpeptidase.

Modifier (mM)	Per cent residual trans-peptidase activity		Per cent residual auto-transpeptidase activity	
	High mol. wt. enzyme	Low mol. wt. enzyme	High mol. wt. enzyme	Low mol. wt. enzyme
None	100	100	100	100
TNBS (1.14)	19	19	26	25
IA at pH 6.9				
1.8	70	70	60	60
18	0	0	13	20
27	0	0	8	8
IA at pH 8.4				
1.8	73	71	60	65
18	0	0	15	25
27	0	0	5	5
DTNB (0.9)	100	100	100	98
PMSF (1.91)	100	98	98	100
Maleic acid (6)	100	100	100	100
PMSF + maleic acid (2)	94	96	73	73
PMSF + maleic acid (4)	81	74	47	40
PMSF + maleic acid (6)	55	41	13	13

glutamyl-*p*-nitroanilide for autotranspeptidase activity (in the absence of glycylglycine) were 17 mM and 9 mM. The activation energy calculated from Arrhenius plots for transpeptidase activity in the temperature range 5–40°C was also comparable for both the high (9300 cal/mole) and low (8600 cal/mole) molecular weight variants of the enzyme. Further, L-serine-borate complex was found to inhibit competitively the transpeptidase activities of both forms of the enzyme (figure 3). The calculated K_i value for L-serine (in the presence of 5 mM borate) were 0.10 mM for the high molecular weight form and 0.17 mM for the low molecular weight form of the enzyme.
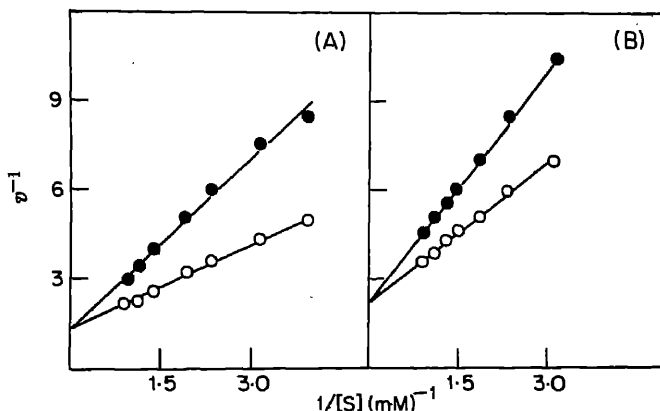
Figure 3. Lineweaver-Burk plots showing competitive inhibition of high molecular weight (A) and low molecular weight (B) variants of GGT by L-serine (0.1 mM) in the presence of sodium borate (5.0 mM). Velocity in the presence of inhibitor, ●; Velocity in the absence of inhibitor, ○.

Discussion

The high molecular weight and low molecular weight forms of urinary γ-glutamyl transpeptidase were found to be similar in many respects. The two variants showed autotranspeptidase and transpeptidase activities, and comparable K_m value for γ-glutamyl-p-nitroanilide, were equally susceptible to serine-borate inhibition and were inactivated to a similar extent by TNBS treatment. However, there were minor differences.

Miller and co-workers (1976) and Tate and Ross (1977) showed that among the different amino acids tested as acceptors with human kidney enzyme L-glutamine was most effective. These workers did not test L-cystine. Shaw *et al.* (1978) later reported that L-cystine was more effective than L-glutamine for the human kidney enzyme. The present studies show that efficiency of acceptors is related to the forms of the enzyme in urine. While the low molecular weight variant utilized L-cystine preferentially as an acceptor, L-glutamine was more effective with the high molecular weight form of the enzyme.

These studies also bring out some essential differences in the transpeptidase and autotranspeptidase activities of the urinary transpeptidase. The present studies show that the optimum pH values for the two activities were respectively 8.6 and 9.4. Shaw *et al.* (1978) reported the pH optima for the two activities for human kidney enzyme as 8.1 and 8.6 respectively. The slight differences could be partly due to the fact that these workers used γ-glutamyl 3-carboxy-p-nitroanilide as substrate whereas in the present studies γ-glutamyl-p-nitroanilide was employed. These workers have also observed a sharper increase of autotranspeptidase activity than transpeptidase activity when the pH increased from 7.0-8.6. While these workers (Shaw *et al.*, 1977) confirmed autotranspeptidation with human serum transpeptidase based on the appearance of a single ninhydrin positive spot in paper chromatography with an R_f value much higher than that of glutamic acid, Orlowski and Meister (1965) demonstrated the formation of di-, tri- and tetra glutamyl-p-nitroanilides during autotranspeptidation with hog kidney transpeptidase. Our results with the urinary transpeptidase are similar to that with serum enzyme in that autotranspeptidation does not appear to proceed beyond the stage of diglutamyl-p-nitroanilide.

It was observed that while IA caused complete abolition of transpeptidase activity at the 18 mM level, 13-25% of autotranspeptidase activity was still retained after this treatment. These data suggest that some of the residues at the active site of the enzyme which are essential for transpeptidase action may not be necessary for autotranspeptidation. IA does not appear to modify the sulphydryl groups of the enzymes since DTNB had no effect on the transpeptidation reaction of GGT variants. It is probable that IA attacks an imidazole residue of the enzyme as in the case of lysozyme (Parson *et al.*, 1969). Recently Elce (1980) has shown that IA can modify a carboxyl group in rat kidney transpeptidase.

Based on the effect of TNBS in the present study, it can be suggested that the amino groups are essential for transpeptidase and autotranspeptidase activities of the two variants. Earlier Elce (1978) has also reported the inactivation of rat kidney enzyme upon modification with TNBS.

The present studies throw new light on the mechanism of action of PMSF-maleic acid on the transpeptidase. Inoue *et al.* (1977, 1978) demonstrated the inactivation of rat kidney enzyme on treatment with PMSF in the presence of maleic acid but not in the presence of PMSF alone. Based on these studies, these workers suggested that maleic acid might enhance the nucleophilicity of the target residue for modification by PMSF. The observation of Tate and Meister (1974) that maleic acid accelerates the enzyme catalyzed cleavage of γ-glutamyl bond tends to support this view. In the present studies, we find that the PMSF-treated enzyme alone did not lose its catalytic activity. However, when maleic acid was added to the enzyme that was subjected to prior treatment with PMSF (excess PMSF was removed by dialysis), there was loss in both transpeptidase and autotranspeptidase activities. These findings suggest that the nucleophile (serine or threonine) of urinary enzyme modified by PMSF is not essential for the activity of the enzyme. However, maleic acid could alter the conformation of the modified enzyme (but not the native enzyme) resulting in loss of catalytic action. PMSF-maleic acid was found to affect the autotranspeptidase activities more than the transpeptidase activities of both the high molecular weight and low molecular weight variants of urinary transpeptidase.

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An acid stable trypsin-chymotrypsin inhibitor from horse gram *(Dolichos biflorus)*

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Abstract. An inhibitor of trypsin and chymotrypsin was purified from horse gram *(Dolichos biflorus)* beans. The concentration of the inhibitor which provided total inhibition was 0.27 µg/µg tryptic enzyme and 0.46 µg/µg chymotryptic enzyme. The inhibitor was stable at 37°C between pH of 3 to 11 and at 97°C, upto pH 5.0 only. While the activities were rapidly lost in 0.1 N NaOH the loss was only 50% in 0.1 N HCl when kept for 2 h at 97°C. On heating at pH 7.8, it remained stable upto 80°C with a gradual loss in activities at 97°C and a total loss occurring by autoclaving at 15 psi for 10 min. Reduction of disulphide bonds by 2-mercapto-ethanol, pronase digestion and boiling in the presence of 1 M NaCl led to reduction in the activities. However, the inhibitor was resistant to the action of pepsin and subtilisin and to urea at 37°C.

Keywords. Trypsin-chymotrypsin inhibitor; horse gram; protease inhibitor; *Dolichos biflorus.*

Introduction

Legume seeds invariably contain inhibitors of proteases. The most widely studied of these inhibitors are the trypsin and chymotrypsin inhibitors (Liener and Kakade, 1970). These inhibitors are generally grouped under three categories — those inhibiting either trypsin or chymotrypsin alone and those which inhibit both trypsin and chymotrypsin. Recently trypsin inhibitors from mung bean (Chrispects and Baumgartner, 1978), Faba beans (Bhatty, 1977) and kintoki bean (Miyoshi *et al.*, 1978) have bean studied. Ray (1970) reported that horse gram *(Dolichos biflorus)*, a commonly used pulse of India, could not support growth in rats but on autoclaving this effect was reversed. The presence of a trypsin inhibitor in the horsegram was suspected. Subbalakshmi *et al.* (1976) found that both the horse gram and moth bean exhibited trypsin inhibiting activity, and this was reduced upon heat treatment. In the light of these reports it was felt useful to purify the trypsin inhibitor from horse gram and to study its properties. In the present study this inhibitor was found to inhibit both trypsin and chymotrypsin.

Abbreviations used: BTEE, benzoyltyrosine ethyl ester; BAEE, benzoylarginine ethyl ester.

Materials and methods

Horse gram was obtained from the local market. Pancreatic α-chymotrypsin (4x crystallised) and trypsin (3x crystallised) were purchased from British Drug House Ltd., Poole, England and Sigma Chemical Co., St. Louis, Missouri, USA, respectively. All other chemicals, enzymes and proteins were from Sigma Chemical Co., St. Louis, Missouri, USA; British Drug House Ltd., Poole, England or E. Merck, Darmstadt, Germany.

Assay of trypsin and chymotrypsin inhibiting activities

(a) *With casein as the substrate:* The method of Kunitz (1948) was used. 0.5 ml of the inhibitor solution was incubated for 15 min at 37°C with an equal volume of trypsin (62.9 μg in 5mM $CaCl_2$) or chymotrypsin (50 μg) in 0.2 M borate buffer, pH 7.8. One ml of 1% casein solution in the same buffer was added and incubation continued for 20 min. Reaction was stopped by the addition of 3 ml of 5% trichloro-acetic acid. The trichloroacetic acid-soluble product in the supernatant was measured by Folin-Ciocalteu procedure, the absorbance at 620 nm providing a measure of enzyme activity. One unit (U) of the inhibitor activity was expressed as decrease by one unit of absorbance measured at 620 nm in 20 min.

(b) *With synthetic substrate:* The method described by Rhodes *et al.* (1960) was followed. A solution of trypsin (62.9 μg in 0.3 ml) or chymotrypsin (50 μg) in 0.004 M acetic acid and 0.02 M $CaCl_2$ was mixed with 0.7 ml of inhibitor solution in 0.006 M Tris-HCl buffer, pH 8.8, followed by 1 ml of 0.006 M Tris-HCl buffer, pH 8.2. After incubation at 37°C for 15 min, 2 ml of substrate-indicator mixture [0.02 M benzoylarginine ethyl ester (BAEE) for trypsin and 0.008 M benzoyltyrosine ethyl ester (BTEE) for chymotrypsin in suitable buffer containing indicator 0.2% *m*-nitrophenol] was added and change in absorbance at 450 nm was measured. The results are expressed as per cent residual enzyme activity.

Estimation of protein

Protein in the fractions from column chromatography was determined according to the method of Lowry *et al.* (1955). Total nitrogen in the lyophilised material was determined by the micro Kjeldahl method of A.O.A.C. (1955), total protein being obtained by using a factor of 6.25.

Polyacrylamide disc-gel electrophoresis

It was done according to the method described by Othmer (1971) using 10% separating gel at pH 7.5 and a constant current of 4 mA per tube for 2 h at room temperature. Two identical gels were run and one was stained with Amido black 10B for protein. The corresponding protein bands from the unstained gel were cut out and protein was extracted with 0.2 M borate buffer pH 7.8. Inhibitory activities were tested in the extracted protein solution.

Activities of trypsin or chymotrypsin complex with inhibitor

The inhibitor (25 μg) in 0.6 ml of 0.006 M Tris HCl buffer pH 8.6 were mixed with 0.3 ml of trypsin (88.1 μg) or chymotrypsin (55.6 μg) solution in 0.004 M acetic acid containing 0.02 M $CaCl_2$ and incubated for 10 min at 37°C to form respective

complexes. To the chymotrypsin-inhibitor or trypsin-inhibitor complex, 0.3 ml
trypsin solution (87.36 µg) or chymotrypsin solution (50 µg) was adde
respectively. It was incubated for 10 min at 37°C before addition of the respectiv
substrate-indicator solution and the absorbance at 450 nm was measured. Enzym
activities were also measured with and without the inhibitors as a control.

Stability studies

For these studies solutions of the inhibitor were prepared in buffers of different pH
values, in 0.1 N NaOH, in 0.1 N HCl, in 1 M NaCl and in 6 M urea and thes
solutions were incubated at temperatures of 37°C and 97°C for different tim
periods. The concentrations of the inhibitor used in these studies were 71 µg/ml fo
antitrypsin activity and 40 µg/ml for antichymotrypsin activity. After coolin
aliquots were removed at various time intervals and pH was adjusted to 7.8 wher
ever needed by addition of 0.2 M borate buffer of suitable pH. The activities wer
measured against trypsin and chymotrypsin using casein as the substrate. Th
buffer systems used in this study were 0.05 M citrate buffer pH 3.0 and 5.0, 0.05 N
borate buffer pH 7.0 and 9.0 and 0.05 M glycine-NaOH buffer pH 11.0.

Digestion with protease enzymes

Solutions of the inhibitor in 0.2 M borate buffer, pH 7.8 were incubated wit
proteases subtilisin BPN and pronase (150 µg enzyme/mg inhibitor) at 37°C fo
48 h. For pepsin digestion, the inhibitor and enzyme solutions were made in 0.06 N
HCl (100 µg pepsin/mg inhibitor) and incubated at 37°C for 48 h. Aliquots wer
removed and placed in boiling water bath for 2 min to inactivate the enzymes an
adjusted to pH 7.8 after cooling before assaying for inhibitor activity. A control wit
heat inactivated enzyme was also run.

Reduction and reoxidation

Reduction of disulphide bond was done as described by Simlot *et al.* (1966) b
dialysing the inhibitor solution against 0.1 M 2-mercaptoethanol for 10
followed by dialysing against 0.05 M borate buffer pH 7.0 in cold. Reoxidation o
reduced disulphide bond was done by exposing the solution to air at pH 8.2 for 1
and 20 h at room temperature.

Results

Extraction of the inhibitor from horse-gram

200 g. of ground horse gram (*Dolichos biflorus*) was defatted with carbo
tetrachloride until the pigment was removed. The cake was dried at 40°C for 24 h t
remove the solvent completely. The defatted meal (100 g) was extracted with 30
ml of 0.2 M acetate buffer, pH 4.2, in cold for 8 h and then centrifuged for 30 min a
900 g. Supernatant was collected and the residue reextracted twice with 125 m
each of the buffer in cold. All the three supernatants were pooled and dialyse
against distilled water for 36 h in cold with changes of distilled water after every 6 h
Antitrypsin, antichymotrypsin activities and protein were determined in th
lyophilised material. The specific antitrypsin and antichymotrypsin activities wer
found to be 8.1 U/mg and 1.8 U/mg protein, respectively.

Preparation of trichloroacetic acid soluble fraction

To the dialysed extract from the previous step, 50% trichloroacetic acid solution was gradually added in cold with continuous stirring till the final concentration of trichloroacetic acid solution was 2.5%. The mixture was kept overnight in cold followed by centrifugation for 20 min at 900 g. The supernatant was decanted off and the precipitate was washed twice with 40 ml each of cold 2.5% trichloroacetic acid solution. Supernatants were pooled and dialysed against distilled water for 40 h in cold with change of water after every 6 h. The dialysed extract was lyophilised and designated as trichloroacetic acid-soluble fraction. The specific antitrypsin and antichymotrypsin activities of this fraction were 25 U/mg and 4.8 U/mg protein, respectively.

DEAE-cellulose chromatography of trichloroacetic acid-soluble fraction

Lysophilised trichloroacetic acid-soluble fraction (30 μg) dissolved in 5 ml of 0.01 M Tris-HCl buffer, pH 8.4, was applied to a column (20 × 1.7 cm) of DEAE-cellulose equilibrated with 0.01 M Tris-HCl buffer, pH 8.4, stepwise elution with the same buffer containing 0, 0.1, 0.15 and 0.2 M sodium chloride was carried out at room temperature. Fifty five fractions of 5 ml each were collected and protein and antitrypsin activity in each fraction was estimated. The result is shown in figure 1A.
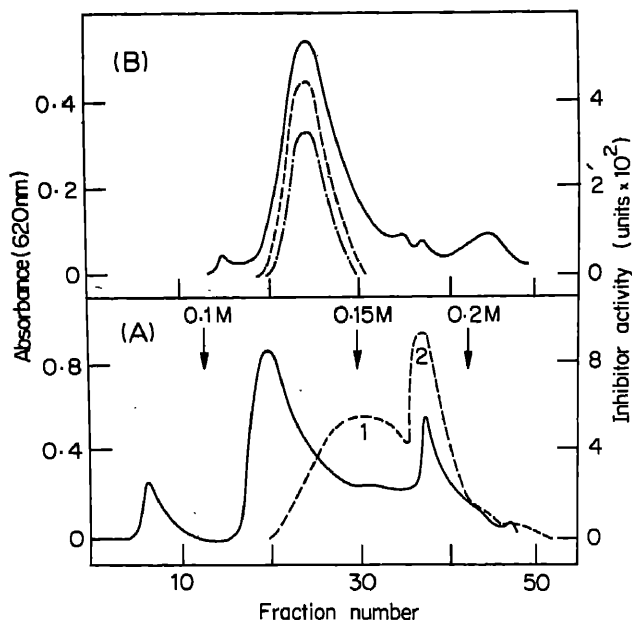
Figure 1. Purification of the inhibitor of trypsin and chymotrypsin.

A) DEAE-cellulose chromatography. Stepwise elution with increasing concentrations of NaCl (0, 0.1 M, 0.15 M and 0.2 M) in buffer 0.01 M Tris-HCl, pH 8.4. Arrows indicate the place of change in eluent. Fraction volume 5 ml/tube.

B) G-50 Sephadex column chromatography of peak-1 from (A) Eluent was double distilled water. Fraction vol. 4 ml/tube.

Protein ———; Trypsin inhibitor - - - -; Chymotrypsin inhibitor (— · —).

Antitrypsin activity was present in two peaks. Fractions corresponding to these were pooled, dialysed against distilled water in cold and then lyophilised. Pooled fractions 24-34 was designated as peak-1 and from 36-40 as peak-2. The specific inhibitory activities against trypsin and chymotrypsin were 68.7 U/mg protein and 13.1 U/mg protein in peak-1 and 43 U/mg protein and 8.5 U/mg protein in peak-2, respectively. Peak-1 with higher specific activity was used for further purification of the inhibitor.

Gel filtration on Sephadex G-50

Lyophilised peak-1 was chromatographed at room temperature on a column of Sephadex G-50 (81 × 1.7 cm) and eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl. Fifty fractions of 4 ml each were collected and this was analysed for protein, antitrypsin and antichymotrypsin activities. When the pooled fractions were dialysed against distilled water in cold prior to lyophilisation, 70% of the total inhibitory activity was found to have been lost. As an alternative, the gel filtration chromatography was then carried out using double distilled water in place of the buffer. The elution pattern with respect to the two inhibitory activities did not change with distilled water and is shown in figure 1B. Fractions corresponding to the peak of antitrypsin and antichymotrypsin activities were pooled and lyophilised. The specific activities of this material were antitrypsin 138.8 U/mg protein and antichymotrypsin 29.7 U/mg protein. This material was used for the study of the properties of the inhibitor. Table 1 records the stepwise purification of the inhibitor. The final purification achieved during the whole

Table 1. Stepwise purification of inhibitor from horse gram.

Steps	Antitrypsin activity				Antichymotrypsin activity			
	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purifi- cation (fold)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purifi- cation (fold)
1. Crude extract	42,770	8.1	100	—	9396	1.8	100	—
2. TCA soluble fraction	8,254	25.0	19.3	3	1578	4.8	16.8	3
3. DEAE-Celluose chromatography								
Peak-1	4,439	68.7	10.4	8.5	847	13.1	9.0	7
Peak-2	1,318	43.0	3.1	5	259	8.5	2.8	5
4. G-50 sephadex chromatoraphy of peak-1	2,891	138.8	6.8	17	619	29.7	6.6	16.5

process was 17 fold for both the inhibitor activities. It may be noted that during each step of purification, the antitrypsin and antichymotrypsin activities remained together suggesting that the inhibitor could be a single component having dual activities.

Polyacrylamide disc gel electrophoresis of the purified inhibitor

Two closely positioned protein bands were observed and both were active against both trypsin and chymotrypsin. The ratio of antitrypsin and antichymotrypsin activities was almost the same (5.2 and 5.4) in both these bands indicating that the inhibitors could be isoinhibitors.

Molecular weight of the inhibitor

Tentative molecular weight of the inhibitor was found to be 13,500 daltons from its elution pattern on a G-50 Sephadex column (not shown) determined according to the method of Andrews (1964). The reference proteins used were alpha chymotrypsiongen (25,000), myoglobin (17,800), ribonuclease (13,700) and cytochrome C (12,400). Elution was carried out with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl.

Inhibition of trypsin and chymotrypsin

Inhibition of the esterolytic activities of the trypsin and chymotrypsin using BAEE and BTEE as the synthetic substrates, respectively, was studied with increasing concentrations of the horse gram inhibitor (figure 2). Even at high concentraions
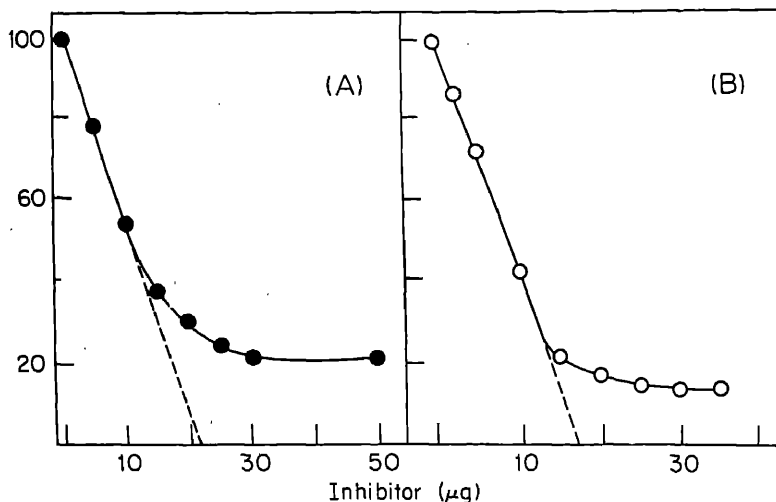
Figure 2. Inhibitor pattern against trypsin and chymotrypsin (for details see text).

A) Chymotrypsin inhibition. Amount of chymotrypsin — 50 µg

B) Trypsin inhibition. Amount of trypsin — 62.9 µg

Broken line denotes the extrapolation of the linear part of the curve for total inhibition.

of the inhibitor, complete inhibition of the enzymes did not occur. 22% of the original alpha-chymotryptic and 13% of the original tryptic activities remained unaffected after treatment with the inhibitor. An equilibrium between the enzyme and the inhibitor appears to have been reached at the high concentration. Extrapolation of linear part of the curves gave values of the amount of inhibitor required to completely inhibit the enzymes. 17.25 µg of inhibitor was required to

inhibit 62.9 µg of trypsin and 22.5 µg of inhibitor was needed to inhibit 50µg of chymotrypsin. On a molar basis inhibitor to trypsin and inhibitor to chymotrypsin ratios came out to be 0.49 and 0.82, respectively.

Activities of inhibitor-enzyme complexes

Preformed inhibitor-chymotrypsin or inhibitor-trypsin complexes when tested for inhibitor activities against trypsin or chymotrypsin, respectively, showed that the inhibitor-chymotrypsin complex inhibited trypsin to the same extent as inhibitor alone whereas inhibitor-trypsin complex inhibited chymotrypsin activity to 60% of that brought about by inhibitor alone (figure 3).
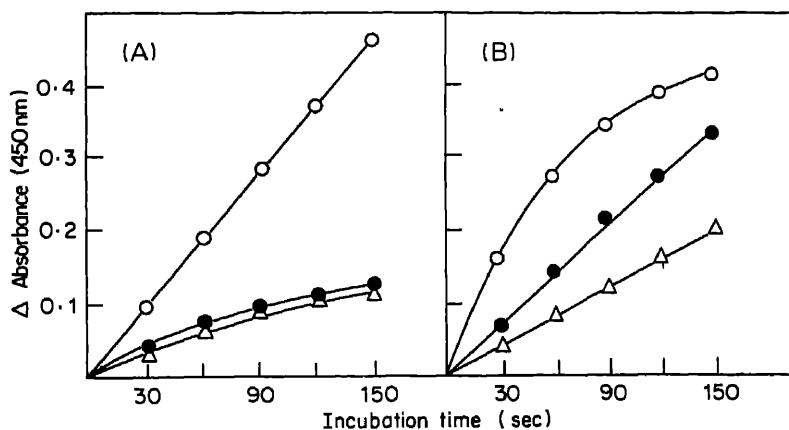
Figure 3. Inhibitor activities of enzyme−inhibitor complexes. Inhibitor was complexed with the enzymes by preincubation with trypsin or chymotrypsin for 10 min in equivalent amounts and the inhibitor activity of the complex was measured (for details see text).

A) Trypsin activity   B) Chymotrypsin activity

Enzyme activity in the absence of inhibitor (O); Inhibition of enzyme by inhibitor (Δ); Activities of the enzyme-inhibitor complexes (●).

Effect of pH

At 37°C, there was no loss of the inhibitor activities in the pH range 3 to 11 and also in 0.01 N HCl up to 48 h of testing (data not shown). In 0.1 N NaOH the inhibitory activity towards both enzymes decreased with time (table 2). At boiling water temperature, the inhibitor was stable at pH 3 and 5 whereas in 0.1 N HCl, a reduction in inhibitory activity was observed (figure 4). At higher pH values the inhibitory activity showed progressive decrease with time. The loss in activity was rapid at pH 11 and in 0.1 N NaOH, 86% and 100% of the activity being lost in 10 min, respectively.

Table 2. Effect of alkali, urea and NaCl upon the activities of the inhibitor at 37°C.

Period of incubation (h)	Inhibition (%)					
	Antitrypsin activity			Antichymotrypsin activity		
	0.1 M NaOH	6 M Urea	1 M NaCl	0.1 M NaOH	6 M Urea	1 M NaCl
0	100	100	100	100	100	100
2	95.5	100	100	91.2	97.8	101.1
6	82.5	100.6	98.7	70.8	93.5	100.6
12	65.0	100.6	98.7	41.6	91.3	101.7
24	39.5	100	98.7	10.9	87.0	101.1
36	25.5	99.4	98.1	0	83.7	101.7
48	20.4	99.4	97.5	0	82.6	100
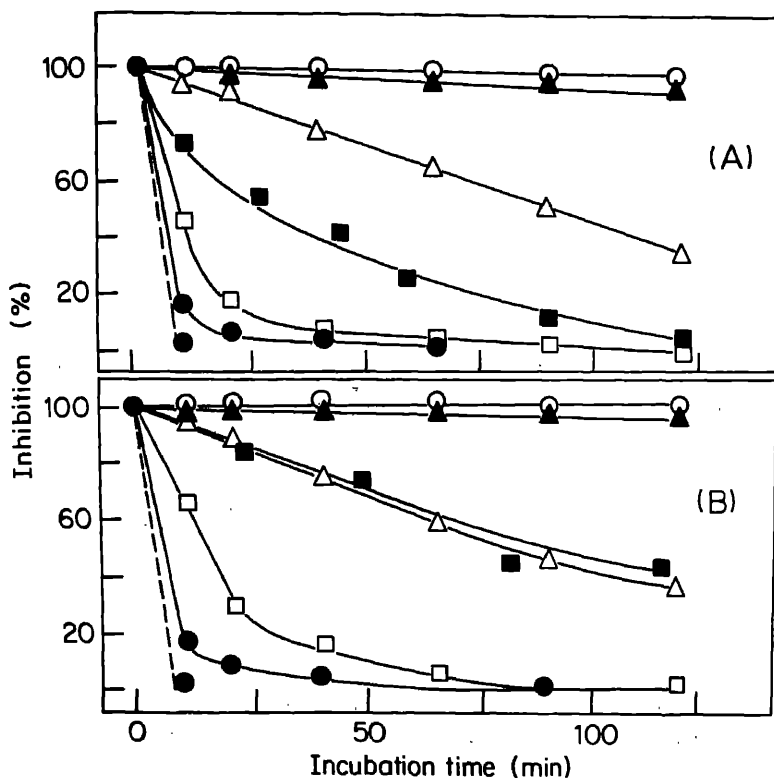
Figure 4. Effect of pH, acid and alkalin on the stability of the inhibitor. Inhibitor was incubated in buffer of different pH and in 0.1 N HCl and 0.1 N NaOH in boiling water and assayed after cooling and bringing the pH to 7.8 (for details see text).

A) Antichymotrypsin activity.   B) Antitrypsin activity.

pH 3.0 (▲); pH 5.0 (○); pH 7.0 (Δ); pH 9.0 (□); pH 11.0 (●); 0.1 N HCl (■) 0.1 N NaOH (--●--).

Effect of temperature

At 40°, 60° and 80°C there was no significant loss of the activities, but at 97°C, there was a gradual decrease in both the inhibitory activities. Autoclaving of the inhibitor solution at 15 psi in 10 min completely destroyed the two activities (figure 5).
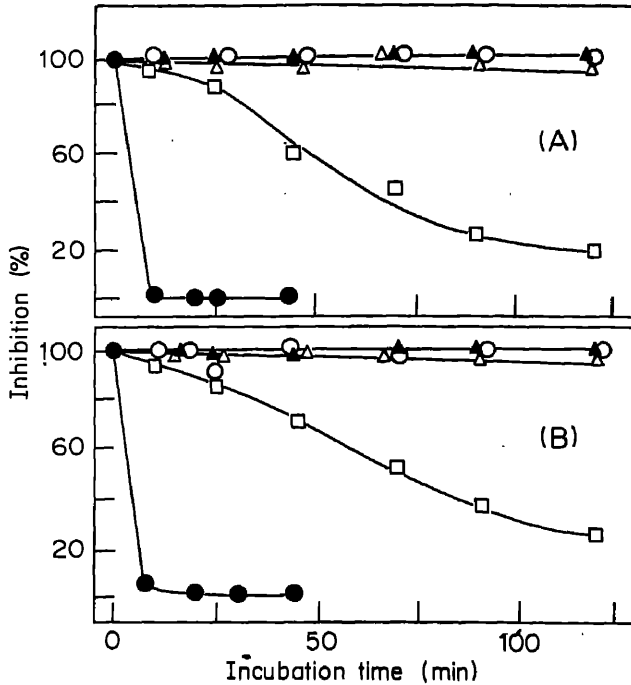
Figure 5. Effect of heating on the stability of the inhibitor (for details see text).

A) Antitrypsin.   B) Antichymotrypsin

40°C (▲); 60°C (○); 80°C (△); 97°C (boiling water) (□); Autoclave at 15 psi (●).

Effect of urea and sodium chloride

At 37°C the loss in inhibitory activity in the presence of urea and NaCl was insignificant upto 48 h testing except 18% loss in antichymotrypsin activity in 6 M urea (table 2). In 6 M urea at 97°C (table 3) there was rapid initial inactivation — 52% loss in antitrypsin activity in the first 25 min and 10% in next 95 min; during the same period the loss in antichymotryptic activity was 30% and 26% respectively. At this temperature the controls themselves showed considerable loss in activity by 95–120 min (table 3).

Digestion with proteolytic enzymes

Digestion with subtilisin had no effect upon antichymotryptic activity, but 20% loss ocurred in trypsin inhibitor activity (figure 6). Similarly pepsin digestion had

Table 3. Effect of urea and NaCl upon the activities of inhibitor at 97°C.

Period of incubation (min)	Inhibition (%)					
	Antitrypsin activity			Antichymotrypsin activity		
	Control	6 M Urea	1 M NaCl	Control	6 M Urea	1 M NaCl
0	100	100	100	100	100	100
10	96	56.2	95.5	92	80	89.1
25	93	47.9	85.8	87	70	70.7
45	80	43.2	75.4	75	64	· 43.5
70	63.5	41.1	53.7	59.5	58	19.6
95	47	38.4	35.8	43	50	8.2
120	36	37.0	26.9	33	44	2.7
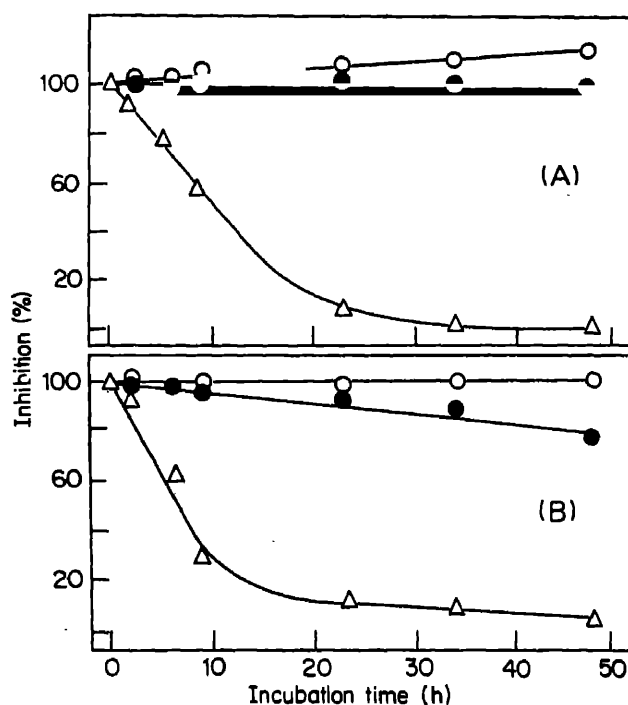
Figure 6. Effect of action of proteases on the activities of the inhibitor (for details see text).

A) Inhibition against chymotrypsin.   B) Inhibition against trypsin.

Pepsin digestion (O); Subtilisin digestion (●); Pronase digestion (Δ).

marginal to no effect upon chymotryptic and tryptic inhibitory activiti respectively. Pronase did inactivate the inhibitor in about 36 h, the relative rate loss of antitrypsin activity was greater than that of antichymotrypsin activity.

Effect of reduction and reoxidation

Upon reduction 69% to 90% of the inhibitory activities were lost (table 4). There was, however, no appreciable reactivation upon reoxidation even after 20 h exposure to air.

Table 4. Effect of reduction and reoxidation of disulphide bonds of inhibitor on its activity.

Activity	Inhibition (%)			
	Control	Reduced	Reoxidised	
			10 h	20 h
Antitrypsin	100	30.9	36.3	38.1
Antichymotrypsin	100	9.5	10.1	10.1

Inhibitor was reduced by dialysing its solution (0.5 mg/ml) in 0.02 M phosphate buffer, pH 7.4 against 0.1 M 2-mercaptoethanol solution in the same buffer for 10 h and then against .05 M borate buffer, pH 7.0 in cold to remove the reagent. Reduced material was reoxidised by exposure to air at pH 8.2 for 10 and 20 h. A control was also run through these steps.

Discussion

An inhibitor purified from horse gram (*D. biflorus*) has been found to inhibit the activity of both chymotrypsin and trypsin. The presence of the two activities in the same molecule was implied by the fact that they could not be separated during the various steps of purification and the two close bands observed on polyacrylamide disc gel electrophoresis contained both the activities in almost the same ratio..The inhibitor activities of the enzyme-inhibitor complexes further supported this.

Trypsin and chymotrypsin inhibitors of plant origin are known for their stability under harsh treatments (Kassel, 1970). The inhibitor from horse gram was resistant to acidic pH (pH 3 and 5) even at high temperature upto 2 h. Inactivation could be obtained only using a combination of strong acid (0.1 N HCl) and high temperature (97°C). At alkaline pH the rate of loss of inhibitor activities depended upon the pH of the solution, temperature and period of incubation. Strong alkali at 37°C and 97°C and autoclaving at 15 psi resulted in rapid destruction of both the activities. Besides being stable to acidic conditions, this inhibitor was also stable to temperatures of upto 80°C at pH 7.8. Even the rate of inactivation at 97°C was rather slow.

Like in the case of inhibitors from soyabean, (Birk, 1961) lima bean, (Jones *et al.*, 1963) kidney bean (Pusztai, 1968) and kintoki bean (Miyoshi *et al.*, 1978) the present inhibitor was also not affected by pepsin action. But pronase, an enzyme of bacterial origin and of broad specificity, could destroy the activities of the inhibitor. It was fairly resistant to the denaturing action of 6 M urea. On the otherhand 2-mercaptoethanol, a disulphide reducing agent, could inactive the inhibitor;

reoxidation however, did not result in recovery of activity. In general, the antichymotrypsin activity was more susceptible to the various treatments compared to antitrypsin activity. The behaviour of horse gram inhibitor was similar to those already reported from various beans in regard to their stability and interaction with trypsin and chymotrypsin. It, however, differed from others in being more resistant to various treatments and in having higher specific activity against trypsin.

Acknowledgement

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Cholesterol metobolism of macrophages in relation to the presence of *Mycobacterium leprae*

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Abstract. Macrophages phagocytose *Mycobacterium leprae* and live bacilli inside such macrophages alter the lipid metabolism. There is increased accumulation of cholesterol ester in the bacteria infected cells. This increase appears to be due to the decreased level of esterase enzyme that could hydrolyse cholesterol esters. Associated with decreased level of this enzyme is the reduced amount of protein synthesis. Increased cholesterol ester may be responsible for conversion of macrophages into foamy cells in the presence of *M. leprae*.

Keywords. *Mycobacterium;* cholesterol; cholesterol ester level; macrophage.

Introduction

Cholesterol is an important lipid component of macrophages and its role has been identified in various structural and functional aspects of the macrophages (Day, 1967; Brown *et al.*, 1980). Macrophages can phagocytose foreign particles, live or otherwise. Accordingly leprosy causing bacilli, *Mycobacterium leprae*, are also taken in by the macrophages. Such an uptake could be shown *in vitro* in macrophage cultures as well as *in vivo* in human tissues from lepromatous leprosy patients. In leprosy patients there is a tendency to develop foamy type of cells and these cells are primarily macrophages which have high lipid contents. Such foamy macrophages are present at sites where there is an infiltration of bacilli and immune competant cells (Yamamoto *et al.*, 1958; Imaeda, 1960; Skinsness, 1970). It was therefore of interest to study lipid metabolism of macrophages in relation to the presence of *M. leprae* which are easily phagocytosed by the macrophage. Kondo and Kanai (1976) had shown that *M. tuberculosis* would induce accumulation of cholesterol ester in macrophages and indicated that this ester could be a major component of the foamy droplets seen in tissue macrophages during tuberculosis inflammation. With this background information it was thought that the behaviour of macrophages in relation to *M. leprae* need to be studied with special reference to lipid metabolism. Since a major alteration was indicated primarily in cholesterol in some of our preliminary experiments, it was chosen as a component of interest.

Material and methods

Microorganism

Mycobacterium leprae were obtained from lepromatous tissue of bacillary positive, treated or untreated patients. Bacilli were prepared as per the method of Ambrose *et al.* (1978). Such isolated bacilli were acid-fast and free from other contaminating bacteria. These do not grow in normal mycobacterial media. The bacilli were counted and 5×10^6 bacilli were added to each leighton tube containing cultured macrophages. *M. leprae* obtained from infected armadillo tissue were also used in some of the experiments. The tissue was supplied by Dr. E. Storrs, Florida, USA.

Collection of macrophage from peritoneal cavity

Macrophages from Swiss white mice were obtained by injecting 5 ml of Eagle's minimum-essential medium + 20% inactivated human AB serum into the peritonial cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected after agitating the cavity and 0.7 ml of the fluid was added to each leighton tube.

Lipid synthesis by macrophages

The macrophages obtained from the peritoneal fluid adhered to the leighton tubes. The medium was changed every 24 h; thereby removing nonadherant cells. After 3 days of such culturing, esterase positive adherant cells were predominately distributed as a uniform layer in the leighton tubes. There were no contaminating neutrophils and non-adherant lymphocytes were not present in significant numbers. Such tubes were infected with *M. leprae* (5×10^6 bacilli/leighton tube). The control tubes did not receive the *M. leprae* inoculum. After 24 h of phagocytosis, the excess bacilli were removed and the macrophages were incubated with [^{14}C]-acetate or [^{3}H]-cholesterol for 4 days for studying uptake and synthesis of lipids.

Extraction and separation of lipids

Following incubation, the macrophages were scrapped off the surface of the glasstubes, the cells counted and the lipids were extracted according to Dole's method (Doles, 1956). Lipids were separated by thin-layer chromatography on 20×20 cm plates of silicia-Gel G (Chemical division, Glaxo Laboratory). The plates were developed at room temperature in a solvent media of hexane : ether : acetic acid (80 : 20 : 1) to separate the lipids. Identification of the lipid spots was made by stainining with iodine vapours, using standard lipids as reference.

Determination of incorporation into lipids

Following identification of the lipid spots, the silica gel corresponding to each spot was carefully scrapped into vials after evaporation of iodine and full decolourisation. Scintillation fluid (10 ml) was added to each vial and radioactivity monitored by using Kontron MR-300 automatic scintillation counter.

[^{3}H]-Cholesterol was obtained as crude tritiated product from Bhabha Atomic Research Centre, Trombay and purified by repeated thin layer chromatography and 0.16 µCi was used for uptake studies. [^{14}C]-Cholesterol oleate (Sp. activity 50.8 µCi/m mol) was obtained from Radiochemical Centre, Amersham and 0.1 mCi was added per leighton tube. [^{14}C]-Acetate 0.5 µCi (Sp. activity 56.7 µCi/m mol) obtained from Bhabha Atomic Research Centre, Bombay, was added in each leighton tube used for incorporation experiment.

Estimation of esterase activity, was carried out according to the method of Vahouny *et al.* (1968). Labelled [^{14}C]-cholesterol oleate (0.2 µCi) in 50 µl acetone was added *via* microsyringe beneath the surface of 2 ml of enzyme preparation containing 200 µmol of potassium phosphate buffer pH 7.4. Incubations were carried out for 1 h at 30°C in a metabolic shaker. Reaction was stopped after 2 h by adding the extraction mixture containing solvents. Lipids were extracted and separated by thin layer chromatography and radioactivity associated with oleic acid and cholesterol ester spots were recorded.

Results

About 60% of the macrophages adherent to the glass had bacilli as demonstrated by the presence of acid fast *M. leprae* inside them. The macrophages in culture with or without *M. leprae* are able to incorporate [^{14}C]-acetate into lipids (table 1). However, macrophages with *M. leprae*, incorporated much less [^{14}C]-acetate into cholesterol. A similar lowered incorporation is also seen, when heat killed *M. leprae* are used as a control. An interesting feature, however, was that the radioactivity associated with cholesterol ester was higher in *M. leprae* infected cultures as compared to the uninfected or those infected with killed bacilli. Data in table 2 present the ratio of acetate incorporated in cholesterol and the ester and it is seen the ratio in macrophages with *M. leprae* is consistently higher. Table 1 also present the ratio of cholesterol ester to cholesterol in each experiment which is higher in the infected macrophages and this increase is statistically significant (P < 0.01). This is reflected in the ratio of ester to cholesterol in all the five experiments (table 1).

When labelled cholesterol was used in incorporation studies it was observed that the total cholesterol uptake was significantly lowered in the bacteria (*M. leprae*) infected cultures (table 3) the ratio of cholesterol ester to cholesterol was also higher in the infected culture compared to the controls.

The increased incorporation into cholesterol ester fraction (monitored by calculating the ratio of incorporation into cholesterol ester : cholesterol) appears to be correlated to size of the *M. leprae* inoculum (figure 1). It is to be noted that only part of the added bacilli get phagocytosed. The uptake of labelled cholesterol and conversion of the label to the ester form have also shown to exhibit different kinetic patterns depending upon whether the macrophage culture is infected or not. While cholesterol uptake increases linearly up to 4 days, conversion to ester appears to be proceeding at maximum rate by 3 days itself. [^{14}C]-Labelled cholesterol uptake by infected cultures showed marked reduction between day one and three of culture, relatively conversion to cholesterol ester did not show much change (figure 2).

Table 1. Acetate incorporation into cholesterol and cholesterol–ester in macrophages with and without *M. leprae*.

(cpm incorporated 10^8 macrophages in 5 separate experiments)

Sr. No.	[14C]-Acetate incorporation (cpm) into lipids			[14C]-Acetate incorporated into cholesterol fraction			[14C]-Acetate incorporated into cholesterol-ester (cpm) fraction			Ratios of incorporation into cholesterol ester: cholesterol		
	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage a	Macro-phage+ heat-killed *M. leprae* b	Macro-phage+ *M. leprae* c
1	3529	3321	722	1392	1145	196	300	240	186	0.22	0.20	0.95
2	3164	3001	1064	372	257	273	413	195	402	1.1	0.75	1.47
3	2050	1950	571	225	143	65	125	85	80	0.54	0.59	1.23
4	3916	3300	1687	3484	2350	1373	760	339	588	0.22	0.14	0.43
5	2723	1675	912	621	358	170	128	123	86	0.20	0.34	0.50

In each experiment 0.5 µCi [14C]-aceate was added to the leighton tube.

p value <0.25   <0.01
         (a–b)   (a–c)

Table 2. Ratio of [^{14}C]-acetate incorporation in cholesterol and cholesterol ester in relation to total uptake of [^{14}C]-acetate (calculated from data of table 1).

Expt. No.	Cholesterol			Cholesterol ester		
	Macrophage	Macrophage+ killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage	Macrophage+ killed *M. leprae*	Macrophage+ *M. leprae*
				a	b	c
1	0.39	0.34	0.27	0.085	0.07	0.25
2	0.11	0.08	0.26	0.13	0.065	0.38
3	0.11	0.073	0.11	0.06	0.043	0.14
4	0.89	0.71	0.81	0.19	0.10	0.35
5	0.23	0.22	0.19	0.048	0.073	0.094

p value—<0.10　<0.01
　　　　　(a–b)　(a–c)

Table 3. [^{3}H]-Cholesterol uptake* by normal and infected macrophages and level of cholesterol ester synthesised inside the cells.

Expt.	Cholesterol level in			Cholesterol ester level in			Ratio of radioactivity in ester/cholesterol in 10^8 macrophages		
	Macrophage	Macrophage+ heat-killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage	Macrophage+ heat-killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage	Macrophage+ heat-killed *M. leprae*	Macrophage+ *M. leprae*
							a	b	c
1	4651	1651	1673	361	156	200	0.078	0.094	0.12
2	2584	1633	1159	394	145	400	0.15	0.09	0.34
3	2050	1647	658	528	230	302	0.26	0.14	0.46
4	6972	3527	814	572	224	216	0.08	0.06	0.265
5	2650	2297	2676	570	408	881	0.21	0.18	0.33

* as cpm radioactivity/10^8 macrophages in five separate experiments　　p value <0.1　<0.0005
　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　(a–b)　(a–c)
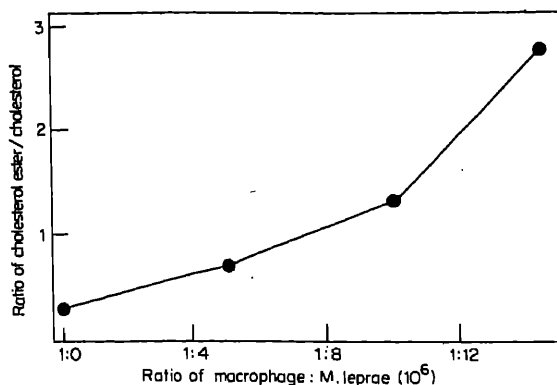
Figure 1. The influence of the number of bacteria, exposed to macrophages, on the ratio of the level of cholesterol ester to cholesterol. The uptake studies were carried out as described in the text using labelled cholesterol.
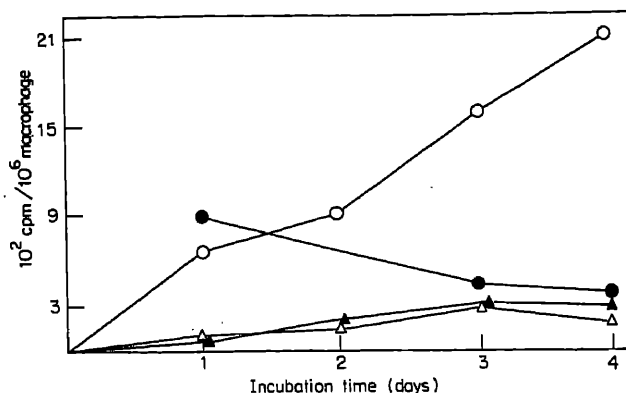
Figure 2. The level of cholesterol and cholesterol ester in macrophages incubated with and without *M. leprae* for various incubation periods in the presence of labelled cholesterol.

(O), Cholesterol in macrophage; (●), cholesterol in macrophage plus *M. leprae;* (Δ), cholesterol ester in macrophage; (▲), cholesterol ester in macrophage plus *M. leprae.*

Since the increased level of ester could be due to reduction in degradation of synthesized ester, the cholesterol ester hydrolytic activity in the infected macrophages was determined. This was done by incorporating into macrophage cultures, [^{14}C]-cholesterol oleate and monitoring the level of hydrolysis in uninfected and infected cultures. The level of hydrolysis is virtually double in uninfected cultures as compared to infected cultures (table 4). This is due to lower level of esterase enzyme in the infected cultures. Using macrophage lysate as a source of the enzyme and [^{14}C]-cholesterol oleate as substrate, the ester hydrolytic activity of infected and control macrophages was determined. The activity was lowered by 50% in the infected macrophages (tabel 4). Thus the macrophage lysate after proper incubation, with or without *M. leprae* was prepared and used to

Table 4. Hydrolytic activity in the macrophages towards cholesterol oleate added to them with or without *M. leprae*.
Radioactivity (cpm) associated with the compounds as calculated/10^6 macrophages.

Expt No.	Cholesterol ester level in		Oleic acid (released)		Per cent hydrolysis*	
	Macrophage	Macrophage + M. leprae	Macrophage	Macrophage + M. leprae	Macrophage a	Macrophage + M. leprae b
1	1844	2040	300	156	14	7.1
2	1086	1700	156	128	12.5	7
3	1020	1810	165	150	14	7.6
4	1360	2192	770	382	36	14.8
5	1082	1999	225	197	17.3	9

* Per cent hydrolysis is calculated as oleic acid released to total radioactivity (ester + oleic acid)

p value—<0.0125
(a–b)

Table 5. Ester hydrolytic activity in the macrophage extract (lysate).

Expt. No.	Cholesterol ester in the reaction mixture*		Oleic acid liberated (as cpm)		Total protein (µg)		Specific activity		Hydrolysis total**	
	Macro-phage	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ *M. leprae*	Macro-phage a	Macro-phage+ *M. leprae* b
1	37590	39540	1680	665	330	205	5	3.2	16	2.9
2	23060	40940	2950	920	630	360	4.7	2.6	16.3	2.2
3	14674	18174	956	274	220	112	4.3	2.4	14.3	4
4	6800	9011	594	660	125	250	4.7	2.8	47	9

* cpm as determined after TLC separation of the ester

p value < 0.0125
(a–b)

** Since the lysate came from different number of macrophages the hydrolysis is standardised to µg protein and 10^6 macrophages.

Specific activity: Oleic acid (cpm) liberated/µg protein.

Total hydrolysis: Oleic acid (cpm) liberated/µg protein/10^6 macrophages.

assay their ability to hydrolyse labelled [^{14}C]-cholesterol oleate *in vitro*. The level of hydrolysis was determined by the amount of oleic acid released by estimating the amount of radioactivity associated with oleic acid separated in TLC from the incubation mixture (table 5). It is clear from the data that quantum hydrolysis by lysate from *M. leprae* containing macrophage, is extremely low compared to the control lysate. The total level of protein is also lower in the lysate from infected macrophages as compared to the control lysate. Nevertheless if specific activity is determined as cpm oleic acid/unit µg protein, and cpm oleic acid/µg protein/10^6 macrophages the enzyme level is again indicated as low in infected macrophages.

Discussion

Macrophages from the peritoneal cavity of Swiss albino mice when cultured *in vitro* show uptake of *M. leprae* as a characteristic phagocytic feature. Phagocytosis of live bacteria could interfere in macrophage metabolism. Earlier Salgame *et al.* (1980) have shown that protein synthesis is reduced in the macrophages when *M. leprae* are present. Present data shows that the lipid metabolism of macrophage are also effected followed bacterial infection resulting specifically in increased cholesterol ester level. This abbreviation in cholesterol metabolism is due to the presence of live *M. leprae* in the macrophage is evident by the fact that heat killed *M. leprae* has no effect. The increase in ester level could be due to either increased synthesis or decreased degradation. The crude extract of the macrophage with or without *M. leprae* was assayed for cholesterol ester hydrolytic activity. This showed that the hydrolytic enzyme (esterase) is very low in *M. leprae* infected macrophage. Interestingly enough such a lowered enzyme level is closely correlated with lowered protein level. Thus we confirm the earlier observation of reduced protein synthesis by Birdi *et al.* (1979) and the system reported for human macrophage by Salgame *et al.* (1980). The reduced enzyme activity is probably a reflection of reduced level of protein synthesis, taking place after *M. leprae* are engulfed by these macrophage. At present we have no data to show the level of cholesterol ester synthetase (ACAT) in these cases, but it appears this enzyme level may not be altered along with lowered protein synthesis. The level of radioactive ester formed when cholesterol is given is not lowered inspite of lower level of cholesterol uptake by *M. leprae* infected cells. This indicate that the synthetic enzyme level may not play a limiting role. It is also clear from all the experiments that the total uptake of cholesterol by *M. leprae* infected cells are lower. We do not know whether it is due to permeability changes after phagocytosis or any other factor affecting the uptake.

There are definite evidences for the role of low density lipoprotein (LDL) for facilitating entry of cholesterol into the cells and in our experiments we presume that LDL present in the human serum enables entry of cholesterol into the macrophages. The basic observation of accumulation of cholesterol ester in macrophages in the presence of *M. leprae* has significant biological implication in the tissue macrophages. It is a common observation that in leproma tissues there are macrophage which have *M. leprae* in them attributed to presence of excess lipids espcially esters. Our experiments with mice macrophages show the reason for such foamy macrophages, is due to accumulation of cholesterol esters. Such ester

accumulation being a clear result of *M. leprae* interaction with host cell is also indicated. Confirmation of similar observations with human macrophage is now being done so as to identify the significance of this phenomenon in leprosy infections.

Acknowledgement

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Dipyrone-induced changes in DNA repair and other cell membrane associated processes in *Escherichia coli*

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Abstract. The analgesic, dipyrone (1,phenyl-2,3-dimethyl-5-pyrazolone-4-methyl amino methane sulphonate sodium), at 20 mM concentration, inhibited the rejoining of single-strand scissions in DNA of *Escherichia coli* B/r cells induced by 20 krad gamma-radiation. The chemical altered the cell membrane structure as evidenced from the uptake of acriflavin, the efflux of potassium ions from the bacterial cells and the inhibition of alkaline phosphatase-a cell membrane associated enzyme.

Keywords. Dipyrone; cell membrane; DNA repair.

Introduction

Those chemicals which exert a reversible effect on the cell membrane could be useful tools in the elucidation of a variety of cell membrane associated processes. We have earlier reported that phenethyl alcohol and procaine hydrochloride affect cell membrane associated process in *Escherichia coli* (Nair *et al.,* 1975; Nair and Pradhan, 1975a). Both the agents are known to alter permeability properties of *E. coli* cells (Nair and Pradhan, 1975a; Silver and Wendt, 1967), rejoining of single-strand scissions in DNA (Nair *et al.,* 1975; Nair and Pradhan, 1975a) and dimerisation of inactive alkaline phosphatase subunits to form active enzyme (Tribuwan *et al.,* 1970; Tribhuwan and Pradhan, 1977). In our search for other chemicals with similar properties, we found that the analgesic and antipyretic drug dipyrone (1,phenyl-2,3-dimethyl-5-pyrazolone,4-methyl-aminomethane sulphonate sodium) caused similar changes in the cell membrane associated functions and inhibited DNA repair in *E. coli.*

Materials and methods

Bacterial strain

Escherichia coli B/r (ORNL) was a stock strain maintained in our laboratory.

Chemicals

Dipyrone was a gift from Unichem Laboratories, Bombay, India. Orthonitrophenyl-β-D-galactopyranoside (IPTG) and paranitrophenyl phosphate (PNP) were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

[Methyl-^{3}H] thymidine (specific activity, 10 Ci/mmol) was obtained from Isotopes Division, Bhabha Atomic Research Centre, Bombay.

Media, irradiation and culture conditions

For DNA sedimentation studies bacteria were grown overnight (18 h) in M9 medium (Lark *et al.*, 1963) supplemented with 0.4% (w/v) glucose, 0.25% (w/v) casamino acids (Difco) deoxyadenosine (50 µg/ml) and [*Me*-^{3}H] thymidine (1000 µCi/ml). The cells were harvested by Millipore filtration, washed with 0.022 M phosphate buffer, pH 7.0 and suspended in the same buffer at the density of 2×10^8 cells/ml. The suspension was irradiated in an atmosphere of nitrogen at 0° C with 20 krad gamma radiation in a Gamma Cell 220 (Atomic Energy Canada Ltd., Ottawa, Canada; 5.2 krad/min). After irradiation, cells were harvested and incubated in M9 medium supplemented with glucose and casamino acids, with or without 20mM dipyrone, for various times at 37°C.

Nutrient broth (Difco) supplemented with 0.5% (w/v) sodium chloride was used for growing the bacteria for studying induction of enzyme and alterations in cell membrane permeability. Viability of the cell populations treated with dipyrone was determined by plating on nutrient agar (Difco) after dilution with M9 medium.

DNA sedimentation analysis

Soon after irradiation or after 1 h post irradiation incubation the cells were converted to spheroplasts by the Tris-EDTA-lysozyme procedure (McGrath and Williams, 1966). The spheroplasts were lysed with 0.5% (w/v) sarkosyl in 0.5 M NaOH on 4.8 ml 5-20% linear gradients of sucrose in 0.1 M NaOH and 0.9 M NaCl. The gradients were centrifuged at 30,000 rpm for 90 min at 20°C using SW 65 rotor in a Spincol L$_2$-65B ultracentrifuge. After centrifugation the gradients were fractionated by siphoning from bottom to top and the trichloroacetic acid-insoluble radioactivity was determined as described earlier and expressed as per cent of total radioactivity loaded on the gradient (Nair *et al.*, 1975; Nair and Pradhan, 1975A). Number average molecular weight (MN), weight average molecular weight (MW) and the number of strand scissions were determined according to Lehnert and Morosona (1971) using a FORTRAN programme in a BESM-6 Computor (Nair and Pradhan, 1975b).

Studies on potassium ion efflux

Bacteria from 18 h nutrient broth cultures were harvested by Millipore filtration and suspended in 0.87% (w/v) sodium chloride solution at a density of 2×10^8 cells/ml incubated at 37°C in the presence of dipyrone at various concentrations for 30 min. The cells were then collected by centrifugation and potassium ion

concentration in the supernatant was determined using a Perkin Elmer 303 Atomic Absorption Spectrophotometer with an air-acetylene flame (Soman *et al.*, 1970).

Studies on acriflavin uptake

The procedure of Silver and Wendt (1967) as described earlier (Nair and Pradhan, 1975a) was used for measuring the uptake of acriflavin by bacteria treated with dipyrone.

Induction of β-galactosidase and alkaline phosphatase

Bacterial cells from 18 h nutrient broth cultures were washed and suspended in phosphate-free medium (A-Pi medium) used by Torriani (1960) containing 1 mM IPTG at a cell density of 2×10^8 cells/ml and incubated at 37°C. After 90 min incubation, dipyrone was added to the final concentration of 20 mM (This was considered as zero time in the figures). The incubation was continued during which aliquots were withdrawn for toluenisation at various time intervals.

Assay of β-galactosidase

β-Galactosidase activity was assayed with ONPG as substrate according to the procedure if Pardee *et al.* (1959) with slight modifications. An aliquot of 0.5 ml toluenised cell suspension was incubated at 37°C with 2 ml of ONPG [1.5 mg/ml in A-P medium (Torriani, 1960)]. After 20 min incubation 1.5 ml of 0.5 M sodium carbonate was added and the absorbance at 420 nm was measured. One unit of enzyme activity as defined here is the amount of enzyme which produces a change of 0.01 $A_{420\ nm}$ units under the assay condition.

Assay of alkaline phosphatase

The assay of alkaline phosphatase was based on the procedure of Torriani (1960). To 0.5 ml toluenised cell suspension 2.5 ml of 0.5 M Tris-HCl pH 8.2 and 0.5 ml aqueous PNP (4 mg/ml) was added and incubated for 30 min at 37°C. At the end of 30 min, 0.5 ml of 10% (w/v) NaOH was added and $A_{420\ nm}$ was determined. One unit of enzyme activity is defined as the activity of the enzyme which produced a change of 0.01 absorbance units at $A_{420\ nm}$ under the assay condition.

Results

Effect of dipyrone on the viability of E. coli

Dipyrone at 20 mM concentration did not show any bactericidal effect both in unirradiated and 20 krad irradiated *E. coli* cells (table 1). This drug at 20 mM concentration did not sensitize the bacteria to anoxic irradiation.

Inhibition of repair of DNA single-strand breaks by dipyrone

The sedimentation rate of DNA derived from *E. coli* B/r cells exposed to 20 krad gamma radiation (figure 1B) is much lower than that of DNA from unirradiated cells (figure 1A). On incubation of the irradiated cells at 37°C for 1 h in supplemented M_9 medium, the sedimentation profile of DNA displayed considerable shift towards that of DNA from unirradiated cells (figure 1C), indicating that most of the single strand breaks induced by gamma radiation were

Table 1. Viability of *Escherichia coli* after exposure to gamma radiation and post-irradiation incubation with 20 mM dipyrone in growth medium.

Radiation dosage	Viability Time of incubation*	
	0 min	120 min
None	8.1×10^7	2.5×10^8
None + dipyrone	8.4×10^7	8.4×10^7
20 krad	4.4×10^7	4.1×10^7
20 krad + dipyrone	4.3×10^7	4.1×10^7

* Total cell count per ml growth medium.
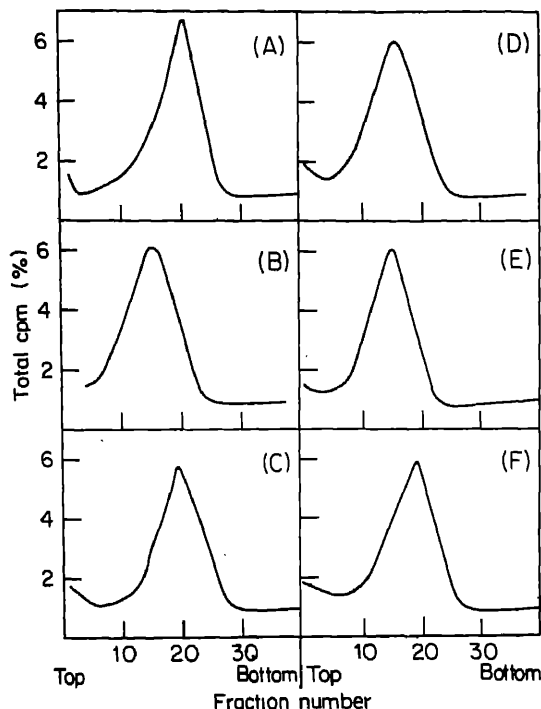
Figure 1. Sedimentation profiles of DNA from gamma irradiated (20 krad) *E. coli* B/r cells after incubation in growth medium with or without 20 mM dipyrone. After prelabelling the cellular DNA with [$_3$H] thymidine, the cells were exposed to 20 krad gamma radiation under anoxia in 0.022 M phosphate buffer, pH 7.0 and incubated with the growth medium at 37°C in the presence or absence of 20 mM dipyrone. The cells were converted to spheroplasts by the Tris-EDTA-lysozyme procedure (McGrath and Williams, 1966) and lysed with 0.5% (w/v) sarkosyl in 0.5 M NaOH on 4.8 ml 5-20% linear gradient of sucrose in 0.1 M NaOH and 0.9 M NaCl. The gradients were centrifuged at 30,000 rpm for 90 min at 20°C using SW 65 rotor in a Spinco L-2-65 B ultracentrifuge and fractionated by siphoning. trichloroacetic acid-insoluble radioactivity in each fraction was determined and expressed as per cent of the total activity loaded·on the gradient.

(A) Unirradiated; (B) 20 krad, 0 min incubation; (C) 20 krad, 60 min incubation in absence of dipyrone; (D) 20 krad, 60 min incubation in presence of 20 mM dipyrone; (E) 20 krad, 120 min incubation in presence of 20 mM dipyrone; (F) 20 krad, 60 min incubation in presence of 20 mM dipyrone; 60 min in absence of dipyrone.

repaired. No such reversal of DNA sedimentation profile of irradiated cells was observed if the medium contained 20mM dipyrone (figure 1D). Prolonging the incubation in the presence of dipyrone for an additional 1 h did not restore the sedimentation pattern of DNA (figure 1E). This chemical, by itself did not introduce strand breaks in DNA since incubation of unirradiated cells with 20 mM dipyrone for 2 h did not lower the DNA sedimentation rate (results not shown). Single strand breaks in DNA of irradiated cell previously incubated with dipyrone for 1 h were repaired upon incubation in growth medium devoid of dipyrone for 1 h (figure 1F), indicating that the inhibitory effect of dipyrone on repair of DNA single strand breaks is reversible.

The MN, MW and the number of strand breaks calculated from the sedimentation data obtained from a typical experiment are presented in table 2.

Table 2. MN and MW of DNA in *Escherichia coli* after exposure to gamma radiation and after post-irradiation incubation under various conditions

Treatments	$MN \times 10^{-7}$	$MW \times 10^{-8}$	Strand breaks per 10^{-8} daltons DNA
A. Unirradiated	4.45	1.80	—
B. 20 krad	3.05	1.08	1.03
C. 20 krad, 60 min incubation	4.18	1.70	0.14
D. 20 krad, 60 min incubation with dipyrone 20 mM	2.94	1.08	1.15
E. 20 krad, 120 min incubation with dipyrone 20 mM	2.94	1.08	1.15
F. 20 krad, 60 min incubation with dipyrone 20 mM followed by 60 min incubation in absence of dipyrone	4.14	1.72	0.17

Changes in cell membrane permeability induced by dipyrone

The effect on cell membrane permeability in *E. coli* was studied by measuring the efflux of potassium ions and the influx of acriflavin. As seen from figure 2, the efflux of potassium ions increased with increasing concentrations of dipyrone. At 100 mM concentration of the drug, the potassium ion efflux reached 40 ppm per 2×10^8 cells.
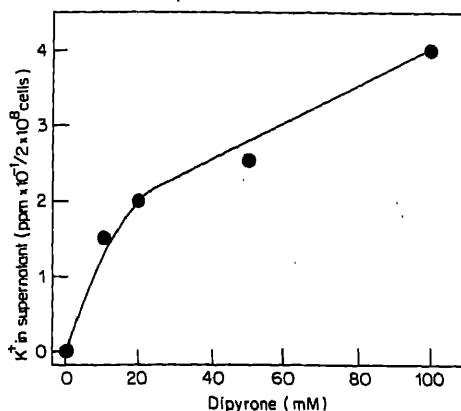
Figure 2. Efflux of potassium ions from *E. coli* induced by different concentrations of dipyrone.

Bacterial cells grown in nutrient broth for 18 h were incubated in 0.87% (w/v) NaCl with different concentrations of dipyrone at a cell densty of 2×10^8 cells/ml for 30 min at 37°. The cells were removed by centrifugation and the potassium ion in the supernatant was measured by atomic absorption spectrophotometry.

As seen in figure 3, incubation of the bacterial cells with dipyrone leads to cellular uptake of acriflavin. At 5 mM concentration of the drug, 5% of the initial amount of acriflavin (0.75 µg/ml medium) was taken up by cells (2×10^8 cells/ml medium), while at 10 mM concentration of the drug, the uptake of acriflavin was 40%. On further increasing the concentration of the drug, there was a graded enhancement in the acriflavin uptake which reached 80% at 100 mM concentration of dipyrone.
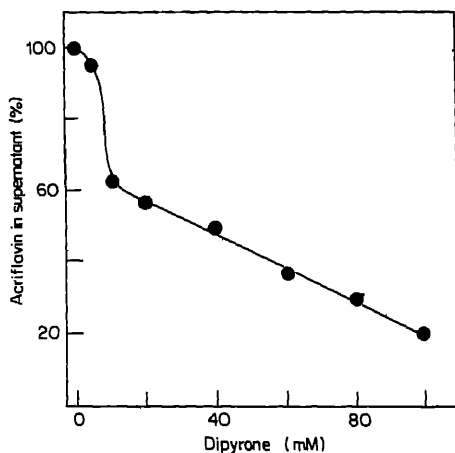
Figure 3. Uptake of acriflavin in *E. coli* cells induced by different concentrations of dipyrone.

E. coli B/r cells from overnight (18 h) nutrient broth cultures were incubated in M_9 medium with different concentrations of dipyrone and acriflavin (0.75 µg/ml) for 30 min at a cell density of 2×10^8 cells/ml. The cells were then removed by centrifugation and acriflavin in the supernatant was determined by fluorimetry.

Effect of dipyrone on induction of β-galactosidase and alkaline phosphatase

Figures 4 and 5 represent the results on the effect of 20 mM dipyrone on induction of β-galactosidase and alkaline phosphatase, respectively. Synthesis of β-galactosidase was only slightly inhibited while the drug completely blocked the synthesis of alkaline phosphatase (both before and after the induction). The slight inhibitory effect on β-galactosidase synthesis could be the reflection of the effect of the drug on overall protein synthesis.
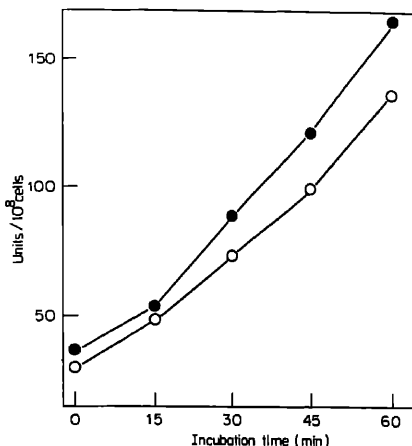
Figure 4. Synthesis of β-galactosidase by *E. coli* cells in presence·of 20 mM dipyrone.

Cells were incubated in A-Pi medium containing 1 mM IPTG for 1.5 h at 37°C. These were further incubated with and without dipyrone. At various times, aliquots of cells suspensions were withdrawn, toluenised and the enzyme activities were assayed.

Control, without dipyrone, (●), with dipyrone 20 mM (O).
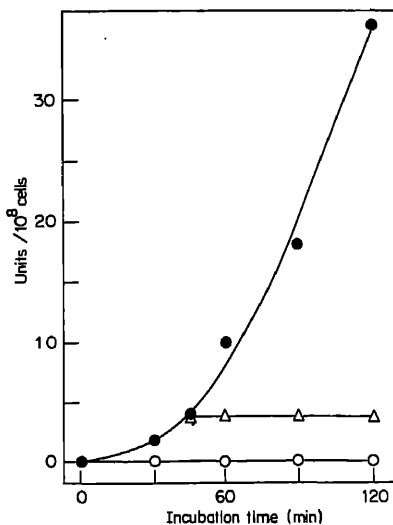
Figure 5. Synthesis of alkaline phosphatase by *E. coli* cells in the presence of 20 mM dipyrone. Details as in figure 4.

Control, without dipyrone (●), with dipyrone 20 mM (O), dipyrone added after induction (Δ).

Discussion

The changes in cell membrane permeability (as seen from altered efflux of potassium ions and influx of acriflavin) brought about by dipyrone indicate that this drug impairs the structural integrity of the *E. coli* cell membrane. It is to be noted that these alterations in the cell membrane structure are reversible. Interestingly the synthesis of alkaline phosphatase—which is shown to be associated with the cell membrane (Tribhuwan *et al.*, 1970; Tribhuwan and Pradhan, 1977a; Schlessinger, 1968) — is also reversibly inhibited by the chemical. The synthesis of β-galactosidase—which is not a cell membrane associated process—is only slightly inhibited by the drug.

The inhibitory effect of dipyrone on the repair of radiation—induced single strand breaks can be inferred as being a consequence of the action of this chemical on the cell membrane. The repair of radiation-induced single strand breaks in DNA of *E. coli* seems to occur by three operationally distinct repair systems: the ultrafast and fast repairs (types I and II) can take place in buffer even at 0°C and the slow repair (type III) can proceed only during incubation in a complete growth medium (Town *et al.*, 1973, 1974). The slow repair is believed to be a multi-step process consisting of the initial cleaning, by exonuclease action of 'dirty breaks' formed in DNA, repair replication in the gaps following excision and ligation of the newly synthesised DNA fragment from the old one (Town *et al.*, 1973, 1974). Our earlier studies have shown that chemicals which alter structural integrity of the bacterial cell membrane block the repair of DNA single-strand breaks (Nair *et al.*, 1975; Nair and Pradhan 1975a).

Dipyrone has a chemical structure (as seen in figure 6) consisting of both hydrophilic and hydrophobic groups. These structural features are thus grossly
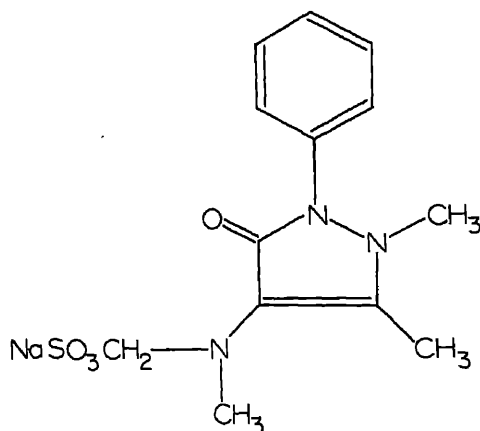
Figure 6. Dipyrone (1, phenyl-2, 3-dimethyl-5-pyrazolone-4 methyl amino methane sulphonate sodium).

similar to phenethyl alcohol and procaine hydrochloride, which were also shown to exhibit inhibitory effects on cell membrane associated process in *E. coli* (Tribhuwan *et al.*, 1970; Tribhuwan and Pradhan, 1977a, b).

Dipyrone thus seems to behave in a fashion similar to procaine hydrochloride and phenethyl alcohol on cell membrane associated processes in *E. coli*. Availability of membrane — affecting chemicals with subtle differences in the mechanisms of their actions amongst them could be useful for studying a variety of cell membrane associated processes including DNA replication and repair. There is a strong likelilhood that these chemicals may have selective actions on the membranes of mammalian cells as well. Indeed the action of procaine hydrochloride on mammalian cell membrane is known and has been studied extensively (Papahadjopolos, 1972; Hauser *et al.*, 1969).

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Increased virulence of *Mycobacterium avium* Kirschberg in thioacetamide treated rabbits

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Abstract. An attempt has been made to determine the correlation between liver damage and the virulence of mildly pathogenic *Mycobacterium avium* in thioacetamide treated rabbits. Liver damage increased the susceptibility of rabbits to infection even with a moderately virulent organism.

Keywords. Liver damage; mycobacteria; virulence; thioacetamide.

Introduction

In an earlier communication (Singh *et al.*, 1980) it was reported that a mild grade of liver damage produced in rabbits by 23 subcutaneous injections of 0.02 ml of carbon tetrachloride (CCl_4) increased the intensity of the disease caused by *Mycobacterium avium* Kirschberg. It was necessary to confirm the finding that liver damage really predisposes the animals to infection more acutely. This was attempted by injecting thioacetamide to produce liver damage in rabbits and challenging them with a mildly pathogenic strain of *M. avium*.

Materials and methods

Materials

Swiss albino outbred mice and rabbits were obtained from the Institute's animal colony and were maintained on standard feeding and watering conditions. The *M. avium* Kirschberg (TMC 801) strain of Mycobacterium was obtained from Trudeau Inc., Sarnac Lake, New Jersey, USA with a reported history of moderate virulence for rabbits and mice.

Methods

The culture of *M. avium* was maintained on Lowenstein-Jensen medium. Thioacetamide (BDH/AR) was weighed quickly and emulsified with 10% tween 80-saline in a mortar and diluted with saline to a concentration of 50 mg/ml. To ascertain the dose of thioacetamide required to produce a mild degree of liver damage in rabbits, mice (5 per group) were administered intraperitoneal injections of 50, 100, 250 and 500 mg/kg of thioacetamide in 0.2 ml quantities. On the basis of

mice experiments, rabbits were injected intraperitoneally with 100 mg/kg of thioacetamide. A weekly repeat of intraperitoneal injection of 25 mg/kg was sufficient to maintain the hepatitic condition of Cameron grade I hepatitis (Cameron and Karunaratne, 1936). Three days after the first injection of thioacetamide, one group of ten rabbits were challenged intravenously with 1 mg of *M. avium* suspended in 0.2 ml of 0.07 M sodium phosphate buffer, pH 8.7. Two groups of 10 animals each were kept as controls. The first group of control rabbits received the same doses of thioacetamide but without challenging with *M. avium*, whereas the other group was kept as normal control with only *M. avium* challenge. Several parameters were recorded like general appearance of the animals, their body weights at weekly intervals and autopsy score of the lesions in the visceral organs *viz.* lungs, liver spleen and kidney. Besides these, weights and Mycobacterial viable counts of the visceral organs, impression smears of the same organs for the presence of acid fast bacilli (AFB) and the mean survival time of the animals after challenge with *M. avium* were also studied.

Results

After 72 h of the first thioacetamide exposure and challenge with *M. avium*, animals of all the three groups [(i) only thioacetamide treated; (ii) thioacetamide treated − *M. avium;* and (iii) normal − *M. avium*] were kept under strict observation. Every animal after death was necropsied to obviate the non-specific deaths. The lesions of the visceral organs provided reasonable assurance that the deaths were due to tuberculosis.

On the death of the first animal in the thioacetamide treated − *M. avium* group on day 17, two animals each from thioacetamide treated – *M. avium* and normal − *M. avium* groups were sacrificed and subjected to detailed studies including the viable counts in the visceral organs. A comparison of various parameters of study is presented in table 1. The thioacetamide-*M. avium* group of animals started deterioration in their general appearance with ruffled hair earlier than the animals of normal-*M. avium* group. Their body weights dropped suddenly while their visceral organs had more intense lesions and increased weights. The impression smears of the organs from this group had an abnormally large number of acid-fast bacteria. On the 17th day of infection, viable counts of the visceral organs in this group were also significantly higher as compared to the control group. The experiment was terminated 91 days after challenging with *M. avium* by sacrificing the surviving animals. The maximum survival time was thus 91 days. The mean survival time of thioacetamide treated-*M. avium* group was only 21.4 ± 0.6 days as compared to 80.2 ± 7.5 days of the normal-*M. avium* group, which is a highly significant change ($t-7.5$, $P<0.001$) (figure 1). All the animals in the thioacetamide-treated but unchallenged group survived till the termination of the experiment. The mean weights of lungs, liver, spleen and kidney of this group of animals were 6.8 ± 0.5, 47.7 ± 3.2, 0.85 ± 0.2 and 9.3 ± 0.3 g respectively which are almost similar to the weights of normal animals of the same weight group (Singh *et al.*, 1980).

Table 1 Comparison of various parameters in the thioacetamide treated and untreated rabbits challenged with *M. avium* Kerschberg. (Figures in parentheses give the range).

Parameters	Thioacetamide treated-*M. avium* group				Normal-*M. avium* group			
	Lungs	Liver	Spleen	Kidney	Lungs	Liver	Spleen	Kidney
CFU/g of tissue[a]	6×10^7	2×10^7	1.8×10^9	4×10^5	8×10^5	1.4×10^8	1.6×10^6	4.0×10^5
Average AFB score[b]	3.5	3	3	2	1.5	1	2	0.5
In impression smears	(3-4)	(2-4)	(2-4)	(1-3)	(1-2)	(0-2)	(1-3)	(0-1)
Average pathological	3	2.5	3	1	1	1	1.5	0.5
score[b]	(2-4)	(2-4)	(2-4)	(0-2)	(0-2)	(0-2)	(1-2)	(0-1)
Mean organ weight g ±SE	19.9±1.4	52.1±5.3	3.1±0.3	15.6±1.5[c]	10.3±0.4	33.2±5.2	1.7±0.3	12.3±0.7[c]
MST (days)[d] ±SE		21.4±0.6					80.2±7.5	
Average body weight (Kg) ±SE								
(a) At infection	1.89±0.12				1.94±0.07			
(b) At death	1.20±0.07				1.60±0.12			

Each value is the mean ±SE of 8 individual observations.

[a] Average of 2 animals. CFU–viable counts

[b] As given in the text. Maximum score = 4.0. AFB–acid fast bacteria

[c] Weights of both kidneys

[d] MST–mean survival time
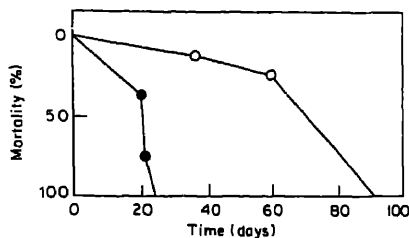
Figure 1. Percentage mortality of normal and thioacetamide-treated rabbits challenged with *M. avium* Kirschberg.

Discussion

An attempt was made to evaluate the susceptibility of the animals after mild degree of liver damage due to infection. This was first tested in carbon tetrachloride treated rabbits (Singh *et al.*, 1980). There was clear indication that liver damage increased the intensity and severity of the disease. CCl_4 in many small subcutaneous doses caused essentially irreversible liver damage. Thioacetamide produce liver damage upon a single intraperitoneal injection but the effect was partially reversible (Gupta, 1956). *M. avium* Kirschberg and rabbit model was chosen with a view that this mycobacterium is mildly pathogenic for rabbits, and produces Yersin type of disease with a big lapse of time. The parameters used to study the extent and severity of pathogenesis of this infection showed that this mild pathogenic organism was severely infective in liver-damaged animals.

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Studies on the conformation of α-globulin from *Sesamum indicum* L. in cationic and nonionic detergents*

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Abstract. The circular dichroic spectra of α-globulin from *Sesamum indicum* L. was recorded in the presence of cetyltrimethyl ammonium bromide, Triton X-100 and Brij-36T. The protein in 0.2 M phosphate buffer pH 7.4 had about 25% β-structure and 5% α-helix, the rest being aperiodic or irregular structure and α-helix, structure was increased by cationic detergent cetyl trimethyl ammonium bromide. But, the increase in α-helix content was much less than that induced by an anionic detergent, sodium dodecyl sulphate. In non-ionic detergent like Brij-36T and Triton X-100, specific β-structures like II-β and I-β were formed along with changes in α-helical and aperiodic structures. These results suggest that the protein has a fairly labile quaternary structure.

Keywords. Circular dichroism; α-globulin; cationic detergent; nonionic detergent; dissociation and denaturation; I-β and II-β structures.

Introduction

The major protein of *Sesamum indicum* L. α-globulin, constitutes ~ 65-70% of the total proteins present in the seed. The molecular weight, the association-dissociation and denaturation behaviour in the presence of various solution conditions of electrolytes, detergents, urea, guanidine hydrochloride, acid and alkali of the isolated fraction was characterized (Nath *et al.*, 1957; Sinha and Sen, 1962; Ventura and Lima, 1963; Prakash, 1976; Prakash and Nandi, 1976 a,b; 1977 a,b,c; 1978; Prakash *et al.*, 1980; Prakash, 1980; Lakshmi and Nandi, 1977, 1978). Recently it was shown that the multimeric α-globulin had a labile secondary structure with a fair degree of conformational freedom and had nearly 70% aperiodic structure, 25% β-structure and about 5% α-helical structure (Prakash, 1980; Prakash *et al.*, 1980). Reagents such as acid, alkali, urea and guanidine

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hydrochloride, caused a significant change in the conformation of α-globulin to more of aperiodic structure as determined by circular dichroism (Prakash *et al.,* 1980). On the other hand, in anionic detergents like sodium dodecyl sulphate (SDS), the protein attained more of ordered structure even at 10 mM SDS (Prakash *et al.,* 1980).

Cationic detergents are used in solubilising membrane proteins (Heller, 1968; Bornet and Edelhoch, 1971; Williams and Gratzer, 1971; Nakaya, *et al.,* 1971; Hong and Hubbell, 1972; Birdi, 1973; Nozaki, *et al.,* 1974; Aoki and Hiramatsu, 1974; and Ushiwata *et al.,* 1975). Also the nature of interaction between nonionic detergents and proteins has received considerable attention in recent years as these reagents also solubilise membrane proteins without disrupting the native structure of the proteins isolated or causing loss of their biological activity (Helenius and Simons, 1971; 1972; Makino and Tanford, 1973; Makino *et al.,* 1973; Helenius and Simons, 1975; Dunnkbey *et al.,* 1976; Shukolynkov *et al.,* 1976; Robinson and Capaldi, 1977; Jirgensons, 1980).

In the presence of cationic detergent cetyl trimethyl ammonium bromide (CTAB), α-globulin was shown to undergo aggregation followed by dissociation and ultimately denaturation (Lakshmi and Nandi, 1977). Also the pK_{Int} of phenolic groups of the protein in the presence of 10 mM CTAB suggested that a conformational change in the protein molecule might have taken place (Lakshmi and Nandi, 1977). On the other hand, in the presence of nonionic detergents Triton X-100 and polyoxyethylene 10-lauryl ether (Brij-36T), dissociation and aggregation of α-globulin occur (Lakshmi and Nandi, 1978). However, no change in the pK_{Int} of phenolic groups of the proteins or the viscosity of the protein was observed even at 1 mM Triton X-100 (Lakshmi and Nandi, 1978) indicating a characteristic difference between the two classes of detergents either in stabilizing or destabilizing the native conformation of α-globulin.

The present study describes work aimed at investigating the conformational stability of α-globulin in solution in the presence of CTAB, Triton X-100 and Brij-36T. Ultimately, this information would help a better understanding of the inter- and intra-molecular forces which hold the various subunits of α-globulin as a thermodynamically stable protein unit.

Materials and methods

Sesame seed of the variety *Sesamum indicum* L. was purchased locally. CTAB, Triton X-100 and Brij-36T were from Sigma Chemical Co., St. Louis, Missouri, USA. All the reagents were of Reagent grade.

Isolation of α-globulin

α-Globulin was isolated by the procedure described earlier (Prakash and Nandi, 1978).

Protein concentration

Protein concentration was determined by using a value of 10.8 for $\varepsilon_{1\,cm}^{1\%}$ at 280 nm (Prakash and Nandi, 1978).

Circular dichroism

Circular dichroic (CD) spectra were recorded with a Cary 60 spectropolarimeter with a model 6001 attachment. Slits were programmed so as to yield a 1-nm band width at each wavelength. The data were reduced to mean residue ellipticites (Θ), using a digital PDP-11 computer and using a value of 115.5 for the mean residue weight for α-globulin calculated according to the amino acid analysis reported elsewhere (Prakash and Nandi, 1978). In the presence of Triton X-100, CD spectra could not be obtained in the near ultraviolet region (360-250 nm) due to the interference of Triton X-100 in the above region as a result of which the noise level was fairly high. The near ultraviolet CD curves in the presence of CTAB and Brij-36T were resolved into gaussian bands by the Du Pont Model 310 curve resolver. The far ultraviolet CD curves were curve fitted using the Digital PDP-11 computer by Greenfield-Fasman procedure (Greenfield and Fasman, 1969). The best-fit curve was taken as the one which had least error and which included most of the experimental points of the far ultraviolet CD curve. A protein concentration of 12.5 μM at various concentrations of CTAB and 5.8 μM at various concentrations of Brij-36T were used for the near ultraviolet CD spectra, using a 1 cm pathlength cell. For the far ultraviolet CD spectra, protein concentration of 12.5, 6.7 and 5.8 μM in the presence of CTAB, Triton X-100 and Brij-36T respectively was used with a 0.01 cm pathlength cell. The spectra were recorded at 25 ± 1°C. The dichroic absorbance differences were averaged from two to three recordings and the mean residue ellipticity was calculated from the averaged spectrum.

All the spectra were obtained in 0.2 M phosphate buffer, pH 7.4. The concentrations of the detergents mentioned is the free concentration of the respective detergents.

Results

The near ultraviolet CD spectrum of α-globulin from 330-250 nm in 0.2 M sodium phosphate buffer, pH 7.4 is shown in figure 1. The spectrum was characterized by a major positive peak at 284 nm with shoulders at 290, 265 and 256-258 nm and a negative peak at 204 nm.

The far ultraviolet CD spectrum of α-globulin from 255 to 200 nm is also shown in figure 1. The spectrum contained a major negative peak around 210 nm. The absence of any fine structure in the spectrum probably indicated the dominance of aperiodic and β-pleated structure in the secondary structure of α-globulin. This is in excellent agreement with the results published earlier (Prakash *et al.*, 1980) and the assignments of the various bands was made as described earlier (Prakash *et al.*, 1980).

In the above procedure, the data is curve fitted by Greenfield-Fasman Procedure (1969) by comparison of the far ultraviolet CD spectra of poly-L-Lysine-Histone standards and/or Yang's standards as the reference spectra. Based on this, the percentage of α-helix, β-and aperiodic structure is calculated for the protein. These values are utilized at best to indicate only the order of magnitude in testing conformational transitions upon variation of solvent systems.
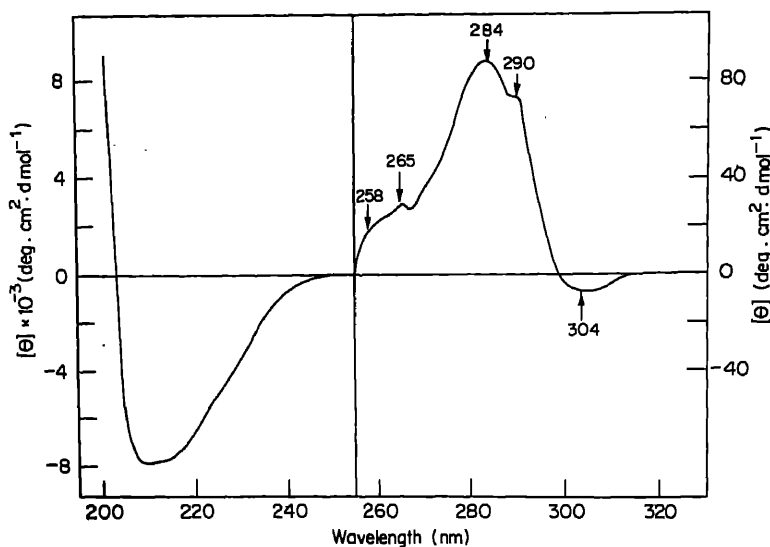
Figure 1. Circular dichroism spectra of α-globulin in 0.2 M sodium phosphate buffer, pH 7.4, in the 190–330 nm region.

An analysis of the far ultraviolet CD spectra of α-globulin in sodium phosphate buffer 0.2 M, pH 7.4 by the procedure of curve fitting indicated that α-globulin consisted of nearly 5% α-helix, 25% β-structure and the remaining aperiodic structure, indicating a similarity in α-globulin structure with the other major storage seed proteins (Jirgensons, 1963a; Jacks *et al.*, 1973; Koshiyama, 1972; and Prakash *et al.*, 1980; Prakash and Narasinga Rao, 1982).

Effect of cationic detergents

The aggregation, dissociation and denaturation of α-globulin in the presence of CTAB has been investigated in detail from our laboratory (Lakshmi and Nandi, 1977). The results indicated that upto a mole ratio of 100 mol of [CTAB] total per mol protein, the protein precipitated out of solution above which redissolution of the protein occurred (Lakshmi and Nandi, 1977). Velocity sedimentation results indicated the presence of higher aggregates in the system at CTAB concentrations of 0.05–1.0 mM. Also the various spectral measurements indicated that the chromophores on the protein were perturbed probably due to conformational change in the protein. In order to look into such conformational changes in the protein, the CD spectrum of the protein was obtained at 0.1, 1.0 and 10 mM CTAB.

Figure 2 shows the near ultraviolet CD spectrum of α-globulin in 0.1 mM CTAB in 0.2 M sodium phosphate buffer, pH 7.4. The spectrum was characterized by a nearly 12% decrease in the intensity of the major peak as compared to that of the protein in phosphate buffer alone. New CD peaks are present at 289, 282, 276 and 270 nm. Also, the fairly sharp negative transition at 304 nm in the control protein is transformed into a broad negative peak spreading from 320–300 nm in the presence of 0.01 mM CTAB. The above results indicate that the tryptophan and
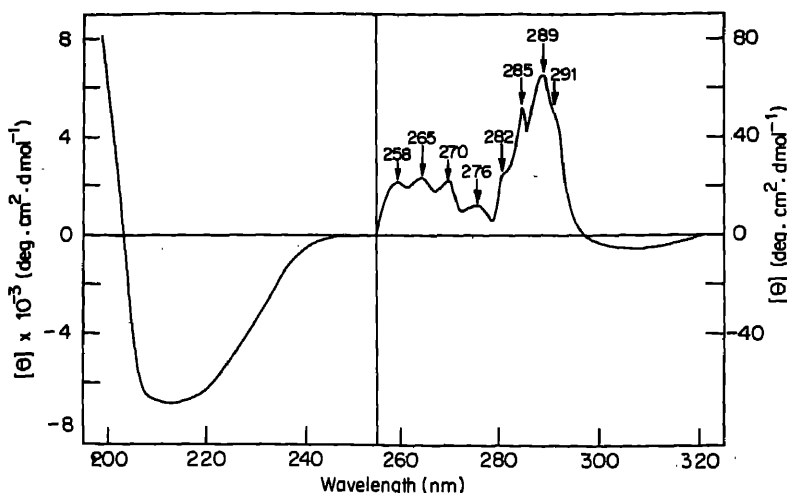
Figure 2. Circular dichroism spectra of α-globulin in 0.1 mM CTAB in 0.2 M sodium phosphate buffer, pH 7.4 in the 190-325 nm region.

tyrosine residues are significantly perturbed at this concentration of CTAB. At this concentration of CTAB, nearly 50% of α-globulin is present as 13 S component in 0.1 M Tris-HCl buffer, pH 8.6, the remaining protein being the dissociated 8 S component and the aggregated 30 S and ~230 S components as observed in velocity sedimentation experiment (Lakshmi and Nandi, 1977). The origin of the above CD spectral change could be due to the association-dissociation of the protein where the tyrosine and tryptophan residue are intimately involved. Further, the significant change in the 304 nm band can be attributed to a more asymmetric environment around the tryptophan residues probably arising out of the binding of charged detergent molecules as well as the change in the microenvironment arising from preferential hydration or change in the dielectric constant of the medium (Strickland, 1974; Prakash *et al.*, 1980). Further, the new peaks in the 250-270 nm region could also arise from the dihedral angle of disulphide, the C-S-S bond angle and the vicinal interactions (Lindberg and Michl, 1970; Timasheff, 1970a, b; Webb *et al.*, 1973; Sears and Beychok, 1973; and Casey and Martin, 1972).

The far ultraviolet CD spectrum of α-globulin under the above mentioned conditions, is also shown in figure 2. A comparison of the far ultraviolet CD spectrum of the protein in sodium phosphate buffer and in 0.1 mM CTAB indicates a decrease in the α-helical content of the protein as also of the aperiodic structure. The curve fitting of the data indicates that the protein has slightly increased α-helical content but the increase is not much as compared to the anionic detergent SDS which induces nearly 20% α-helix at 1.0 mM SDS (Prakash *et al.*, 1980). This has been observed with other proteins also (Jirgensons, 1967; Verpoorte and Kay, 1966; Lederer, 1968; Ikeda and Hamaguchi, 1970; Timasheff, 1970a, b; and Viser and Blout, 1971). The percentage of α-helix, β- and aperiodic structure is given in table 1. No decrease in aperiodic structure is indicated in the curve fitting procedure, this may also be due to the compensatory effect of the β-structure.

Table 1. Secondary structure of α-globulin at 24-26°C under various solvent conditions[a]

No.	Solvent	Structure (%)		
		α-Helix	β	Aperiodic
1.	Sodium phosphate buffer, pH 7.4, 0.2 M	5	25	70
2.	CTAB (mM)			
	0.1	10	16	74
	1.0	11	13	76
	10	5	10	85
3.	Brij-36T (mM)			
	0.1	15	15	70
	1.0	10	9	81
	10	10	21	69
4.	Triton X-100 (mM)			
	0.01	10	12	78
	1.0	10	12	78
	10	13	8	79

[a] Estimated by curve fitting to far ultraviolet CD spectra using Greenfield-Fasman procedure (1969).

The near ultraviolet CD spectrum of α-globulin at 1.0 mM CTAB is shown in figure 3. From the figure it is apparent that there was a further decrease in the amplitude of the overall CD spectrum. The above results in conjunction with the results at 0.1 mM CTAB concentration indicated a further change in the spectral properties of the chromophores at this concentration of CTAB.
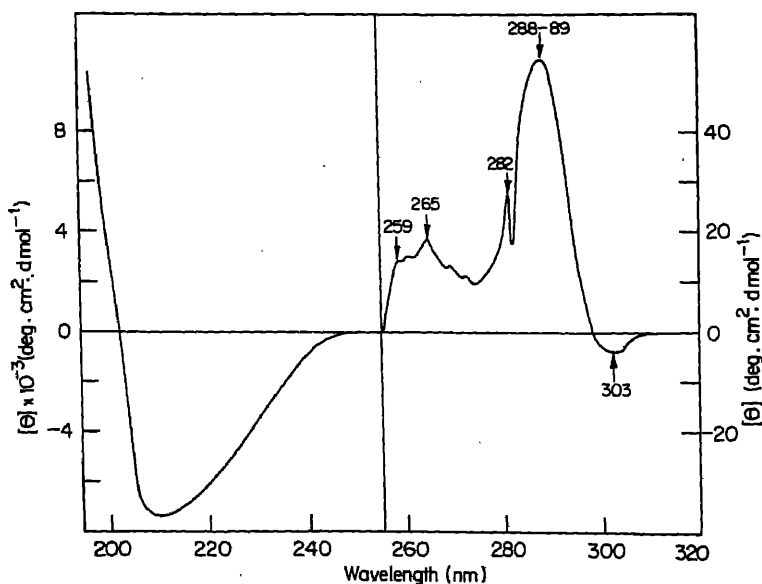
Figure 3. Circular dichroism spectra of α-globulin in 1 mM CTAB in 0.2 M sodium phosphate buffer, pH 7.4 in the 190-320 nm region.

The far ultraviolet circular dichroic spectrum is also shown in figure 3. The spectrum looks every similar to that at 0.1 mM CTAB except that the 210 nm band was shifted to 209 nm and was less broad. The curve fitting of the data indicates 76% of aperiodic structure, 13% β-structure and 11% α-helix structure.

The near ultraviolet CD spectrum of α-globulin at 10 mM CTAB concentration is shown in figure 4. The amplitude of the peaks remains the same as at 1.0 mM CTAB. However, there was a much better resolution of the 290, 287, 275, 266 and 258 nm peaks. Also the negative peak at 304 nm was blue-shifted to 302 nm. At a
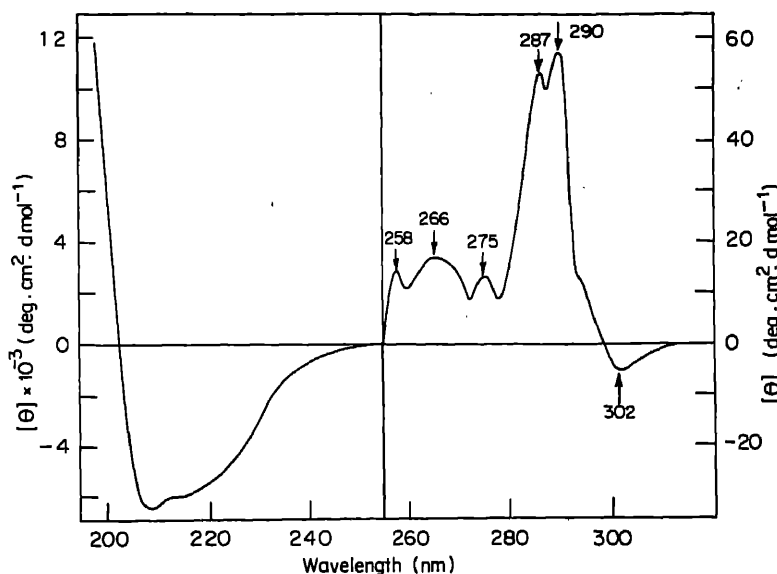
Figure 4. Circular dichroism spectra of α-globulin in 10 mM CTAB in 0.2 M sodium phosphate buffer, pH 7.4 in the 190-320 nm region.

similar concentration of SDS, the protein was devoid of any CD bands in the near ultraviolet region indicating a gross difference in the conformation of α-globulin in the presence of anionic and cationic detergents (Prakash *et al.*, 1980).

Figure 4 also shows the far ulraviolet CD spectrum of α-globulin in presence of 10 mM CTAB. A small change in the amplitude at 210 nm is observed and 210 nm original CD is blue-shifted to 208 nm band indicating increased aperiodic structure in the protein. The curve fitting of the data showed that nearly 85% of the protein was of aperiodic structure, 12% β-structure and 5% α-helical structure. A comparison of the spectra indicated a decrease in the α-helix structure of the protein though not significant, and could possibly be envisaged in various kinds of aggregates that are found at this concentration of CTAB. However, the predominant structure at this concentration of CTAB was aperiodic as compared to SDS where at a similar concentration, the protein has nearly 20% α-helix, 10% β-structure, the remaining being aperiodic (Prakash *et al.*, 1980).

Effect of non-ionic detergents

The nonionic detergents Triton X-100, Brij 36T (polyoxyethylene 10 lauryl ether) was shown to dissociate and aggregate α-globulin above critical miscellar concentration of the detergent (Lakshmi and Nandi, 1978). Spectroscopic measurements indicated no change in the environment of the tryptophan and tyrosine inspite of dissociation and aggregation of the protein in the presence of either Triton X-100 or Brij-36T (Lakshmi and Nandi, 1978). Binding measurements suggested that perhaps micelles of the detergent bind predominantly to the exposed hydrophobic surfaces of the protein subunits. However, viscosity measurements showed no major conformational change of the protein in the detergent solutions (Lakshmi and Nandi, 1978). Hence it was of interest to look into the near and far ultraviolet CD spectra of α-globulin in the presence of the above nonionic detergents to obtain an insight into the conformational stability of the protein in the presence of these detergents. This was of special interest, since as mentioned earlier, these detergents are known neither to disrupt the native structure nor cause any loss in their biological activity (Thompson and Bachelard, 1970; Alexander and Penefsky, 1971).

(A) Brij-36T

The near ultraviolet CD spectrum of α-globulin in 0.01 M Brij-36T is shown in figure 5. The CD spectrum was very similar to that of the CD spectrum of α-globulin in sodium phosphate buffer alone except that the negative trough at 304 nm was broader. This observation is in excellent agreement with the spectrophotometric and fluorescence measurements where no change has been
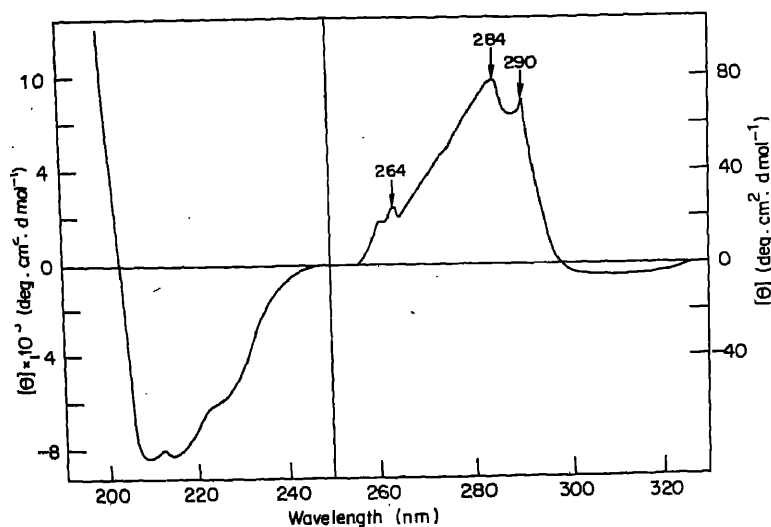
Figure 5. Circular dichroism spectra of α-globulin in 0.1 mM Brij-36T in 0.2 M sodium phosphate buffer, pH 7.4 in the 190-325 nm region.

observed in the environment of tyrosine and tryptophan (Lakshmi and Nandi, 1978). However, the small change of the asymmetric environment around the

tryptophan residues due to the binding of detergent molecules producing a change in the preferential hydration might be responsible for the alteration in the CD trough at 304 nm.

Figure 5 also shows the far ultraviolet CD spectrum of α-globulin in the presence of 0.01 M Brij-36T. The peaks had a larger intensity as compared to that of the protein in buffer alone. Also, the fine structure of the spectra was enhanced with a CD band appearing as a shoulder at 226 nm and well resolved troughs at 209 nm and 215 nm indicating the generation of a more ordered structure in the protein. The curve fitting analysis showed 15% α-helix, 15% β-structure and the balance being aperiodic structure (table 1). Although a nonionic detergent like Brij-36T is known not to disrupt the native structure in general (Thompson and Bachelard, 1970; Alexander and Penefsky, 1971), in α-globulin, increased ordered structure was induced with a concomittant decrease in the β-structure. Stevens *et al.* (1968), based on their studies on the circular dichroism of polypeptide films concluded that for the I-β-structure, the spectrum displays a positive band between 196 and 200 nm and a negative band between 216 and 220 nm and for the II-β-structure, the CD spectrum is shifted to higher wavelength and is particularly pronounced in the longer wavelength negative band at ~ 228 nm (Timasheff *et al.*, 1967). However, the contributions, if any, from the aromatic side chains should induce a positive band in the 230-250 nm region (Woody, 1978). Although α-globulin is rich in tryptophan, tyrosine and phenylalanine (Prakash, 1976; Prakash and Nandi, 1978), it is probable that the 227 nm band of α-globulin in the presence of 0.01 M Brij-36T is due to anti-parallel β-structure in the protein. However, during the curve fitting procedures, this is also evaluated in terms of α-helical and β-structures.

Figure 6 shows the near ultraviolet CD spectrum of α-globulin in 1 mM Brij-36T. There was a decrease in the amplitude of the spectrum and generation of a
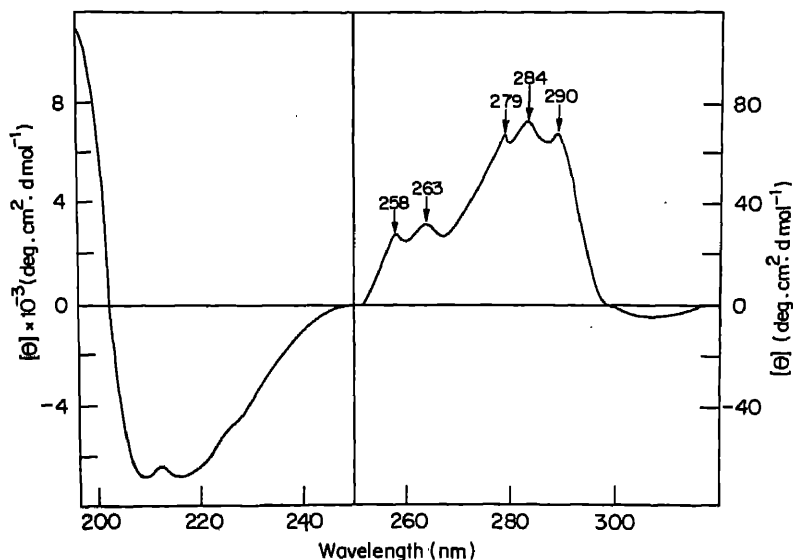
Figure 6. Circular dichroism spectra of α-globulin in 1 mM BRIJ-36T in 0.2 mM sodium phosphate buffer, pH 7.4 in the 190-320 nm region.

new CD band at 279 nm. The new CD band at 279 nm indicated a slight perturbation of the tyrosine and/or tryptophan residues at this concentration of the detergent. In most cases, the positive peaks at 264 nm and 256 nm is attributed to phenylalanine arising from (0-0) and (0-932 cm^{-1}) transitions, respectively (Strickland, 1974). It is quite possible that the blue-shifted peak at 263 nm is an indication of the perturbation of phenylalanine residue by the detergent molecules. However, as mentioned earlier, such an explanation would not rule out the CD bands arising due to the dihedral angle of disulphide, the C-S-S-bond angle and the vicinal interactions (Lindberg and Michl, 1970; Timasheff, 1970a, b; Webb *et al.*, 1973; Sears and Beyehok, 1973; and Casey and Martin, 1972).

The far ultraviolet CD spectrum of the protein in presence of 1.0 mM Brij is also shown in figure 6. The spectrum is characterized by a decrease in the negative intensity of the peak and also, the resolution of 227 nm band has decreased, probably indicating the formation of more of an aperiodic structure in the system. The curve fitting analysis of the spectrum indicates nearly 81% aperiodic structure, 10% α-helix and 9% β-structure in the protein. At this stage, it is worthwhile noting that the protein undergoes aggregation as well as dissociation without involving tyrosine and tryptophan residues (Lakshmi and Nandi, 1978).

Figure 7 shows the near ultraviolet CD spectrum of α-globulin in 10 mM Brij-36T. In the near ultraviolet spectrum the 283 nm band becomes fairly broad with
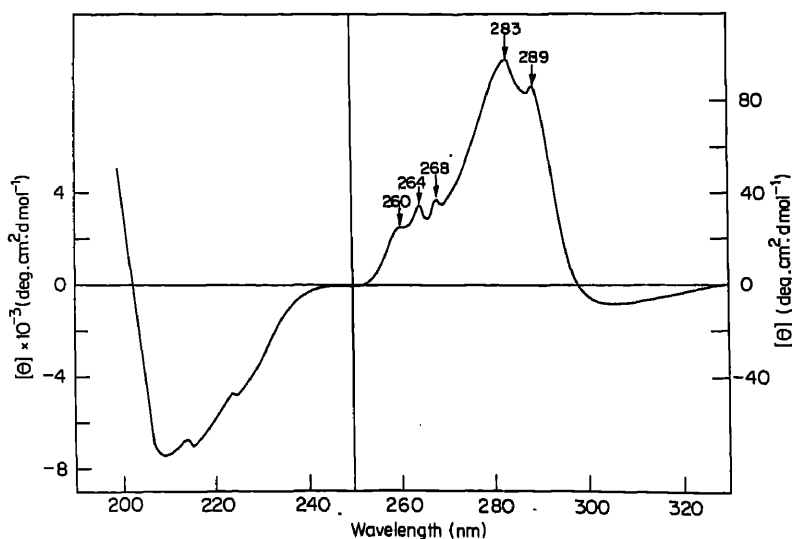
Figure 7.   Circular dichroism spectra of α-globulin in 10 mM BRIJ-36T in 0.2 M sodium phosphate buffer, pH 7.4 in the 190-330 nm region.

the elimination of 279 nm band present at 1 mM Brij. The above results indicate that at this concentration of the detergent, there is probably more perturbation of the phenylalanine residue (of course with the constraint of disulphide dihedral angle and the C-S-S-bond angle) as compared to that of the tyrosine and

tryptophan residues. These results are in conformity with the already observed results of the protein at 10 mM Brij in terms of the absence of participation of tyrosine and tryptophan residues in the association-dissociation phenomenon of α-globulin (Lakshmi and Nandi, 1978).

The far ultraviolet CD spectrum is also shown in figure 7. The intensity of the spectrum remains the same as that of the CD spectrum of protein in 1 mM Brij. The CD bands at 209-210 and 227 nm become more pronounced as compared to those at 1.0 mM. The curve fitting analysis of the spectrum shows that nearly 10% of the protein structure consists of α-helix, 21% β-structure and 69% aperiodic structure indicating the formation of more of β-structure in the protein which appears to be the trend as was discussed earlier. This concentration of 10 mM Brij, is higher than the critical micellar concentration of the detergent and results have to be interpreted with much caution.

(B) Triton X-100

In figure 8 are shown the far ultraviolet circular dichroic spectra of α-globulin in presence of 10 µM, 1 and 10 mM Triton X-100, respectively. The spectrum at
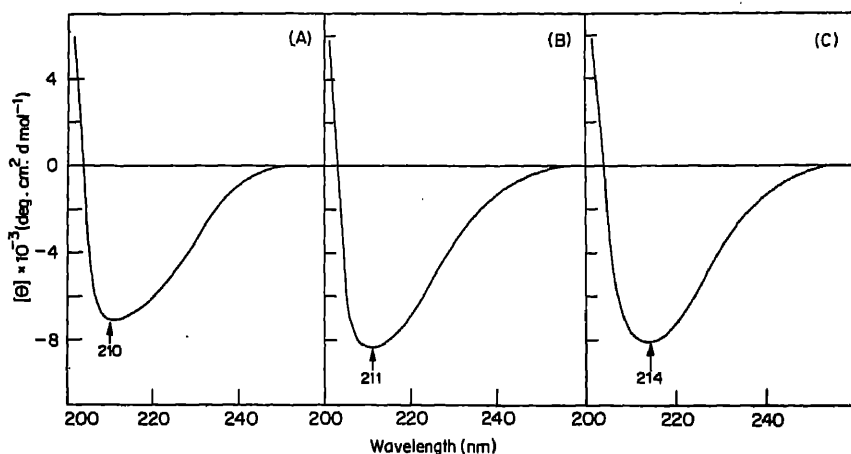
Figure 8. Far ultraviolet circular dichroic spectra of α-globulin in 10 µM, 1 and 10 mM Triton X-100 in 2.0 M sodium phosphate buffer, pH 7.4, in the 200-260 nm region.

10 mM Triton is very similar to that of the protein in buffer alone, with minimum at 210 nm; however the intensity of the spectrum is decreased. The curve fitting analysis indicates an increase in aperiodic structure to nearly 78% with a corresponding decrease in the β-structure. On the other hand, at higher concentration of Triton X-100 (1.0 mM), the percentage of aperiodic β- and α-helical structures remain the same, but the trough has a minimum at 211 nm instead of 210 nm and is broader towards larger wavelengths. However, at this concentration of Triton, the intensity of the trough is increased as compared to the 10 µM triton spectrum and is almost equal to that of the control protein in buffer alone, probably indicating the formation of more ordered structure. At still higher

concentration of Triton X-100, i.e., at 10 mM, there is a significant shift in the trough minima to 214 nm. The curve fitting analysis showed nearly 13% α-helix, 8% β-structure and 79% aperiodic structure as indicated in table 1. Apparently even at 10 mM Triton X-100 which is above the critical micellar concentration nearly 21% ordered structure is present in the protein. Stevens *et al.* (1968) have shown that the circular dichroic spectra of β-structured polypeptides of the category I, termed as I-β display a strong negative band between 210-220 nm very similar to the optical rotatory dispersion patterns of a number of poly-amino acids (Fasman and Potter, 1967). In view of these results the broad negative band of the protein at 214 nm which shifted from 210 nm in presence of 10 mM Triton X-100 might have arisen from the parallel β-structure or I-β structure. Obviously the broad nature of the CD band indicates that it is a combination of aperiodic, β and α-helical structures. However, the point that is relevant is that the shift of 4 nm in the protein peak from 10 mM Triton to 10 mM triton is probably due to the formation of I-β-structure in the protein in the presence of the detergent Triton X-100.

Discussion

The circular dichroism spectra of the α-globulin under various solution conditions of cationic detergents like CTAB or nonionic detergents like Brij-36T or Triton X-100 indicate that considerable variation is exhibited in the secondary structure of α-globulin in terms of α-helical, β-structure and the aperiodic structures. Makino *et al.* (1973) have emphasised that the use of neutral detergents and the bile salt anions, cholate and deoxycholate, can often extract proteins from membranes without disruption of the native conformation or loss of biological activity in contrast to commmon synthetic ionic-detergents, notably dodecyl sulphate which ordinarily solubilise lipid-associated proteins in inactive and denatured form. The effect of the anionic detergent, SDS, on the conformation of α-globulin has been investigated in detail earlier (Prakash *et al.*, 1980). The protein unlike many of the lipid-bilayer membrane proteins, undergoes different kinds of conformational transitions in all the three kinds of detergents, viz., in anionic, cationic and neutral detergents. As reported earlier, in SDS, the protein has more ordered structure in terms of α-helix structure even at 10 mM SDS concentration (Prakash *et al.,* 1980). On the other hand, in cationic detergents like CTAB along with α-helix and aperiodic structure the protein also has different conformations like antiparallel β-structure as compared to the native protein where no such structure could be identified. At these concentrations of CTAB, Lakshmi and Nandi (1977) have observed aggregates of the protein which accompanies perturbation of the tryptophan and tyrosyl residues. On the other hand, in their studies on the effect of nonionic detergents on the physico-chemical properties α-globulin they have concluded that micelles of the detergent predominantly bind to the exposed hydrophobic surfaces of the protein subunits (Lakshmi and Nandi, 1978). Keeping in view the observed effect of CTAB, on the near ultraviolet CD spectra, which is significant, it is understandable that tyrosyl, tryptophanyl and phenylalanine residues are involved in the process. Even assuming that many groups of tryptophan, tyrosine and phenylalanine are exposed to the solvent (though

thermodynamically not feasible), the observed effect of CTAB which is quite significant cannot be explained. Hence some of these aromatic residues must be exposed to the bulk solvent as a result of conformational change of the protein. This is confirmed by the far ultraviolet circular dichoric spectra of α-globulin in presence of various concentrations of CTAB. In fact at lower concentrations of CTAB more of α-helix is induced in the system which decreases slightly at 10 mM SDS nearly 20% of the protein was present in the form of α-helical structure (Prakash *et al.*, 1980). These induced secondary structure of α-globulin in CTAB in fact decreases at higher detergent concentration. Mattice *et al.* (1979) have reported that the class of trimethylammonium chloride detergents are an exception to the usual ability of ionic detergents to promote formation of ordered structures in oppositely charged homopolypeptides. They have observed that the CD of fully ionized poly L-aspartic acid, is nearly unaffected by dodecylammonium chloride, whereas, the detergent destablizes the helical form of poly L-glutamic acid, such a disorganization of the tertiary structure of the protein by CTAB is expressed in a decrease of the CD bands related to the tyrosine and tryptophan chromophores and also the appearance of the vibronic fine structure in the phenylalanine band zone in α-globulin (see results). Helenium (1971) has observed that the lipid-free protein obtained with SDS or CTAB had altered immunological properties. Our observations of the effect of SDS and CTAB on the induced conformational states of α-globulin might have a bearing in explaining similar results.

However, neutral detergents have been shown to have an advantage that their availability in free concentration is automatically limited by their low critical micellar concentration, such that cooperative binding and denaturation cannot occur for any reasonable excess addition of the detergents. However, this does not exclude the possibility of a change in the conformation of protein in presence of neutral detergents possibly leading to a loss of activity or association-dissociation phenomenon in the protein. The results in the present study with Brij-36T in the near ultraviolet region indicate that the spectral perturbation of the protein cannot be directly correlated with the change in the secondary structure of the protein. In the near ultraviolet region at various concentrations of Brij-36T only the vibronic transitions of phenylalanine residue is altered significantly. At this concentration of the detergent the protein has been shown to undergo aggregation and dissociation (Lakshmi and Nandi, 1978). Looking at these results it is quite possible that some of the phenylalanine residues are in the contact areas of the subunits or are entropically trapped or released during the aggregation or dissociation of the protein respectively. In the far ultraviolet region, as compared to the ionic detergents, where there is an induced α-helix conformation, in presence of Brij-36T along with the already present α-helix region, a new CD band is observed at 227 nm which has been attributed to the II-β or anti-parallel β-structure (see results). The above result is interesting in the sense that these nonionic detergents not only are active in generating probably some α-helix structure in the system but also in generating new kinds of structures that probably were not present in the native structure in a significant amount. Probably these II-β-structures are a pre-aggregation or pre-dissociation step leading either to aggregation or dissociation. On the other hand, in Triton X-100 even though it is also a nonionic detergent a

different CD band is observed at 214 nm which is attributed to the I-β structure more akin to the parallel β-structure. Further, Lakshmi and Nandi (1978) have observed that in Triton X-100 also the protein undergoes an association-dissociation phenomenon above cmc of the detergent. This means that the secondary structure of the protein either I-β or II-β by itself is not probably responsible for the aggregation and dissociation of the protein directly.

The analysis of the CD spectrum of α-globulin in presence of various detergents reported in this paper can serve as a good example of the serious limitations which exist in the interpretation of the circular dichroism spectra of various proteins under varying solvent conditions in terms of specific types of conformations. In particular, at present it is still a difficult task to assess quantitatively the contributions made by side-chain chromophores to the far ultraviolet circular dichroism spectra inspite of the several studies available on this subject. Further, a practical limitation also arises from the inadequacy of polypeptide models or proteins whose structures are well established as models to predict quantitatively the circular dichroism contributions from specific conformations. However, it has been possible to analyse some of the unusual features of the far ultraviolet CD spectrum of α-globulin in terms of theoretical considerations about the α-helix spectrum and the variability in terms of the contributions of β-structures specially I-β and II-β-structures.

Acknowledgements

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Partial specific volumes and interactions with solvent components of α-globulin from *Sesamum indicum* L. in urea and guanidine hydrochloride*

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Abstract. The interaction of α-globulin with urea/guanidine hydrochloride was investigated by determining the apparent partial specific volumes of the protein in these solvents. The apparent partial specific volumes were determined both under isomolal and isopotential conditions. The preferential interaction parameter with solvent components calculated were 0.08 and 0.1 g of urea and guanidine hydrochloride respectively per g protein. In both the cases the interaction was not preferential with water. The total binding of denaturant to α-globulin was calculated both for urea and guanidine hydrochloride and the correlation between experimentally determined number of mol of denaturant bound per mol of protein and the total number of peptide bonds and aromatic amino acids were found to be in excellent agreement with each other. The changes in volume upon transferring α-globulin from a salt solution to 8 M urea and 6 M guanidine hydrochloride were also calculated.

Keywords. Partial specific volume; denaturation; preferential interaction; α-globulin; urea and guanidine hydrochloride.

Introduction

Urea and guanidine hydrochloride (GuHCl) are classical protein denaturants and their use has been extensive. GuHCl has been frequently used as a subunit dissociating agent for multimeric proteins. Urea, although less effective, is still used extensively as a protein denaturant. The mechanism of denaturation of proteins by urea and GuHCl and the thermodynamic aspects of this reaction have been the subject of extensive studies and the present state of knowledge has been summarised by Tanford (1968, 1970) and Pace (1975).

α-Globulin is the major storage protein of sesame seed, *Sesamum indicum* L. and constitutes nearly 65-70% of the total proteins present in the seeds. The protein has been-isolated and characterized and some of its properties studied by various physico chemical techniques (Nath *et al.*, 1957; Sinha and Sen, 1962;

Abbreviations used: GuHCl, Guanidine hydrochloride; PS buffer, 0.1 M phosphate buffer, pH 6.5 containing 1 M NaCl.

Ventura and Lima, 1963; Prakash, 1976; Prakash and Nandi, 1976, 1977a, b; Prakash, 1978; Prakash, 1980; Prakash *et al.*, 1980; Prakash, 1981 a, b; Prakash, 1982; Lakshmi and Nandi, 1977, 1978). The weight average molecular weight of the protein has been estimated to be $274000 \pm 20,000$ by sedimentation equilibrium in phosphate buffer, 0.1 M, pH 6.5 containing 1 M NaCl (Prakash, unpublished results). It is shown to consist of at least 12 subunits (Prakash and Nandi, 1978).

The partial specific volume, $\bar{V}$, of a protein in a particular solvent is a parameter which is necessary for the determination of the molecular weight of the protein from sedimentation equilibrium data, as well as for the interpretation of small angle x-ray scattering results. In practice, this quantity, $\bar{V}$, is frequently assumed or calculated from amino acid composition. A small error in the partial specific volume can lead however, to a large error in the calculated molecular weight ultimately leading to erroneous conclusions on the stoichiometry of assembly of macromolecules and also to serious errors in the calculation of the various thermodynamic parameters of associating systems. In particular if the experiment is performed in concentrated denaturant solutions such as 6 M GuHCl or 8 M urea the problem becomes more serious due to a large change in partial specific volume of proteins in these solvents. Although several methods are currently used for the measurement of partial specific volume like pycnometry, density gradient columns (Linderstrom-Lang and Lanz, 1935; Hvidt, *et al.*, 1954; Reithel and Sakura, 1963), H_2O-D_2O exchange technique (Edelstein and Schachman, 1967; Schachman, 1957), magnetic float method (Ulrich *et al.*, 1964) and precision densimetry (Stabinger *et al.*, 1967), the last two methods are used more extensively in the determination of the $\bar{V}$ of proteins due to their high precision and ease of operation. The measurement of the partial specific volume as a thermodynamic parameter can reflect and give an insight into protein denaturation, and its contribution is two fold. First, through changes in the volume of the protein upon denaturation; second, through interactions which the protein may undergo with the solvent component *i.e.* preferential interaction either with water or with the denaturant. It is feasible to measure both the preferential interaction parameter as well as volume change upon denaturation by determining the partial specific volume of the protein under various thermodynamic conditions.

α-Globulin has been shown to undergo association-dissociation in the presence of various additives like electrolytes, detergents, urea, GuHCl, acid and alkali. With some of the reagents, the association-dissociation process is also accompanied by denaturation and conformational changes in the protein (Prakash, 1976; Prakash and Nandi, 1976, 1976a, b, 1978; Prakash *et al.*, 1980; Prakash, 1981; Lakshmi and Nandi, 1977, 1978). Of particular interest were urea and GuHCl, in the presence of which α-globulin initially undergoes aggregation and dissociation and ultimately denaturation (Prakash and Nandi, 1977b) leading to 100% aperiodic structure (Prakash *et al.*, 1980). Hence it was of interest to look into the thermodynamics of the interaction of these denaturants with α-globulin. The present paper describes the measurements of the preferential interaction parameters, volume changes upon denaturation and the calculations of the various thermodynamic parameters in 8 M

urea and 6 M GuHCl solutions to get an insight into the dynamics of the multimeric protein α-globulin, in these solvents.

Experimental procedures

Materials

Extreme purity GuHCl from Heico Inc., USA was used after purification as described under Methods. Ultrapure urea from Schwarz/Mann was used after purification as described under Methods. Reagent grade sodium phosphate, sodium acetate, methylamine hydrochloride, phosphorus pentoxide and acetic acid were from Fischer Scientific Co.,USA. All other chemicals were of Reagent grade and were used without further purification.

Methods

Purification of GuHCl: GuHCl dissolved in water was passed through a 40 μ pore size millipore filter system and the ultraviolet absorbance of this solution was measured in a Cary 118 spectrophotometer against deionized water from 240–400 nm. Only solutions which had less than 0.05 absorbance at 240 nm and no absorbance between 260–400 nm were used.

Purification of urea: A concentrated solution of urea in water was treated with activated charcoal and stirred for 4 h. The solution was filtered through Whatman filter paper No. 44 to remove coarse char coal particles and further filtered twice through millipore filter and to the filtrate methylamine hydrochloride was added (final concentration-0.012 M) and stirred well. The solution was passed through a column previously gravity packed with Bio-Rad Analytical grade mixed bed resin, AG 501-X8(D) 20-50 mesh, fully regenerated. The eluate concentration of urea was measured both densimetrically and refractometrically with water. The final concentration of urea was adjusted to 8 M with proper buffer salts to have a 8 M urea solution in buffer as described elsewhere (Prakash *et al.,* 1981b).

Preparation of α-globulin solution in dilute buffer

α-Globulin was isolated in a homogeneous form by the previously described method (Prakash and Nandi, 1978). A solution in 0.1 M phosphate buffer, pH 6.5 containing 1 M NaCl (PS buffer) was prepared.

Preparation of α-globulin solution in 8 M urea buffer or 6 M GuHCl

The partial specific volume of α-globulin in 8 M urea buffer and 6 M GuHCl was measured (i) at constant molality and (ii) at constant chemical potential as described elsewhere (Prakash *et al.,* 1981b).

For measurements at constant molality, 5-25 mg sample of the protein was weighed into acid-cleaned test tubes and dried again at 40°C over phosphorus pentoxide for at least 56 h. Air was admitted into the vacuum oven by bubbling it through a concentrated sulphuric acid trap. Immediately after taking the protein out of the oven, an aliquot of urea or GuHCl solution pre-eqilibrated to room temperature was added and the test tubes were sealed with parafilmquickly. The protein was allowed to dissolve slowly without vigorous shaking. The tubes were

kept in a humidity-controlled constant temperature room at 20°C for 4 h. Then the tubes were centrifuged at 15,000 g for 20 min at 10°C and any evaporative losses were minimised. These tubes were equilibrated overnight at 20°C before isomolal measurements were done. This time of equilibration permitted elimination of any air bubbles from the solution, since with experience it was found that microbubbles caused serious problems in density measurements. The same solutions were used for concentration measurements by ultraviolet absorbance and for the measurement of V at constant chemical potential, conditions. For routine measurements at constant chemical potential, the protein solutions were dialyzed at 4°C against 250 ml of 8 M urea buffer or 6 M GuHCl for 2-3 days and at 20°C against 250 ml of PS buffer for measurements in buffer alone. The last 24 h of dialysis in the presence of urea or GuHCl was performed at 20° C, which is the temperature at which the density measurements were performed. Thermal equilibrium of the samples were established as described elsewhere (Prakash *et al.,* 1981b).

Determination of protein concentration: Protein concentrations were measured routinely by ultraviolet absorption. For the accurate determination of the extinction coefficient the deionized protein (~2 g) was dissolved in water and again dialyzed overnight against deionized water. The protein solution was then centrifuged at 100,000 g for 1 h to remove any undissolved materials and suspended aggregates or particles in the solution. It was then filtered through a millipore filter; from the filtrate an aliquot was then transferred to the 8 M urea in buffer or phosphate buffer containing 6 M GuHCl such that the protein absorbance was well within the range of 1.0 absorbance unit. After 4 to 5 h the ultraviolet absorption spectrum of the solution was obtained in triplicate from 250 to 400 nm in a Cary 118 spectrophotometer. The light scattering corrections were made as described elsewhere (Prakash *et al.,* 1981b). The average of the ultraviolet absorbance of the three samples was then determined. A known volume (~300-400 ml) of the above stock protein solution was freeze-dried and then dried under vacuum at 40°C in the presence of phosphorus pentoxide for 72 h. The exact weight of the protein in each sample was obtained from the difference in weight between the predried, tared flask prior to the addition of the protein solution and after exhaustive drying of the protein. The extinction coefficient of the protein was calculated from a knowledge of the corrected ultraviolet absorbance in the buffer or in 8 M urea buffer or in 6 M GuHCl solution. The values of the extinction coefficients for α-globulin at 280 nm in various solvents are: 0.2 M phosphate buffer pH 6.5 containing 1 M NaCl, 9.54 dl/g-cm; 8 M urea buffer pH 4, 8.94 dl/g-cm and 6 M GuHCl, 8.93 dl(g-cm).

Handling of protein solutions for density measurement: Extreme care was taken to avoid foaming of the protein solution during transfer to any container or to the densimeter as also to minimize evaporation. The solutions from the test tubes were drawn slowly and carefully with a disposable 1 ml syringe. The needle was then replaced by a female luer adapter to facilitate the transfer of the solution to the densimeter. For measurements at constant chemical potential, the dialysis bag was retrieved from the dialyzing system with stainless steel forceps and the solution was taken out of it with a 1 ml disposable syringe and needle and immediately transferred to the densimeter.

Density measurements: The densities of water, the solvent and the protein solutions of various concentrations were measured with a precision Densitymeter DMA-02 (Anton Paar, Gratz), as described by Lee and Timasheff (1974a).

The density of each solution was measured with reference to a known standard. The difference between the densities of two samples is given by:

$$\rho_1 - \rho_2 = (1/A)\ (T_1^2 - T_2^2) \tag{1}$$

where A is the instrument constant determined with solutions of known density, ρ_1 and ρ_2 are the densities of the known and unknown solutions and T_1 and T_2 their respective time periods as read directly from the display of the densimeter. All measurements were made at 20°C with the densimeter cell compartment maintained at $20° \pm 0.02°C$ with a refrigerated and heated form Scientific Company Circulating water bath with a large reservoir, the entire system being kept in a chamber where humidity was controlled and maintained at 20°C.

Analysis of data: The densities of the solvent and the protein solution were converted to an apparent partial specific volume ϕ using the following equation (Schachman, 1957, Kielly and Harrington, 1960, Cassassa and Eisenberg, 1961, 1966).

$$\phi = \left(\frac{1}{\rho_0}\right)\left\{\ 1 - [(\rho - \rho_0)/c]\ \right\} \tag{2}$$

where ϕ is the apparent partial specific volume, ρ and ρ_0 are the densities of the solution and solvent, respectively in g per ml, and C is the concentration of protein in g per ml. The obtained values of ϕ, were then plotted as a function of protein concentration and the extrapolated value at zero protein concentration was taken as the partial specific volume $\bar{V}_2^0$. For three component systems, the notation of Scatchard (1946) and Stockmayer (1950) was adopted setting components, 1, 2 and 3 as water, protein and the added diffusible material, *i.e.*, urea or GuHCl.

The preferential interaction parameter

$$\left[\frac{\partial g_3}{\partial g_2}\right] T_1\ \mu_1,\ \mu_3 = \xi_3 \tag{3}$$

of the solvent components with the macromolecule was then calculated from the proper combination of densities obtained at constant chemical potential and constant composition of solvent components (Cohen and Eisenberg, 1968).

$$\left[\frac{\partial g_3}{\partial g_2}\right] T,\mu_1,\mu_3 = \left\{\left[\frac{\partial \rho}{\partial g_2}\right] T_1\ \mu_2\ \mu_3 - \left[\frac{\partial \rho}{\partial g_2}\right] T,P,m_3 \right\}\Big/\left[\frac{\partial \rho}{\partial g_3}\right] T,P,m_2 \tag{4}$$

where T is the absolute temperature, P is pressure, μ is the chemical potential of the particular component i, g is the concentration expressed as of i per g of principal solvent, m is the mol of component i per 1000 g of water and ξ_3 is preferential

interaction parameter. From the definition of the partial specific volume, at infinite dilution, the above equation may be re written as

$$(-\phi'_2 \rho_0)^0 = (1 - \phi_2 \rho_0)^0 + \xi_3 (1 - \overline{V}_3 \rho_0) \tag{5}$$

where, ϕ'_2 and ϕ_2 are the partial specific volumes measured at constant chemical potential and constant solvent molality respectively. The superscript o indictates the values corrsponding to infinite dilution of the macromolecular species. In practice, however, it was possible to use ϕ_2 measured at the given concentration since generally it was found that the apparent partial specific volumes were independent of protein concentration.

The volume change upon transferring a negative protein to a denaturing environment is given by (Eisenberg, 1976)

$$\Delta V = M_2 \; (\Phi_1 - \overline{V}_2) \tag{6}$$

where M_2 is the molecular weight of the protein, and $\overline{V}_2$ is the partial specific volume of the native protein in dilute buffer and ϕ_1 is the partial specific volume in urea or GuHCl under constant molality.

Strictly speaking, the above value of ΔV, does not correspond just to the volume change of the protein itself upon unfolding, but it is also a function of the contribution from all other volume changes, such as differences between the changes of volume of solvent components when they interact with the protein, difference in electrostriction in the two media and volume changes associated with changes in ionization of protein side chains upon denaturation.

Calculation of the number of mol of denaturant bound per mol of the protein

The apparent partial specific volume of the protein, at infinite dilution of the macromolecular species, is related to the preferential interaction parameter (Lee and Timasheff, 1974a)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3} \simeq \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu,\mu_3} = \xi_3 \tag{7}$$

by

$$\phi'_2 = \phi^*_2 - \xi_3 \left(\frac{1}{\rho} \phi V_3\right) \tag{8}$$

where $\overline{V}$ is the partial specific volume of the protein in dilute buffer and the other symbols have their usual notation as described before. The above equation is valid since (Cassassa and Eisenberg, 1966; Cohen and Eisenberg, 1968).

$$\xi_3 = \left[\left(\frac{\partial \rho}{\partial g_2}\right)_{\mu_3} - \left(\frac{\partial \rho}{\partial g_2}\right)_{m_3}\right] \Big/ \left(\frac{\partial \rho}{\partial g_3}\right)_{m_2} \tag{9}$$

The preferential interaction parameter is related to the actual amount of solvent components bound to the protein by (Noue and Timasheff, 1972).

$$\xi_3 = A_3 - g_3 A_1 \tag{10}$$

where all interactions are expressed in units of g of ligand bound per g of protein; A_3 is absolute solvation, *i.e.* the actual amount of denaturant bound to the protein; A_1 is the absolute hydration, and g_3 is the solvent composition, expressed as g of denaturant per g of water. A combination of equation (8) and (10) gives

$$\phi'_2 - \phi^*_2 - (\frac{1}{\rho} - \overline{V}_3)(A_3 - g_3 A_1) \tag{11}$$

from which the apparent partial specific volume of the protein chemical equilibrium with solven φ'_2 can be calculated if

$$\phi^*_2, A_3, A_1, g_3, \text{ and } \overline{V}_3 \text{ are known.}$$

Calculation of A_3: The extent of urea or GuHCl binding to protein in 8 M urea of 6 M GuHCl is calculated according to Lee and Timasheff (1974b) and Prakash *et al.* (1981a) for GuHCl and urea respectively.

Results and discussion

The partial specific volume of α-globulin were determined under both isomolal and isopotential conditions in (i) PS buffer (ii) 8 M urea buffer and (iii) 6 M GuHCl solution.

(i) PS buffer

The concentration dependence of apparent partial specific volumes is presented in figure 1 for α-globulin in PS buffer. The isomolal value at zero protein concentration was 0.728 ± 0.002 and the isopotential value at zero protein
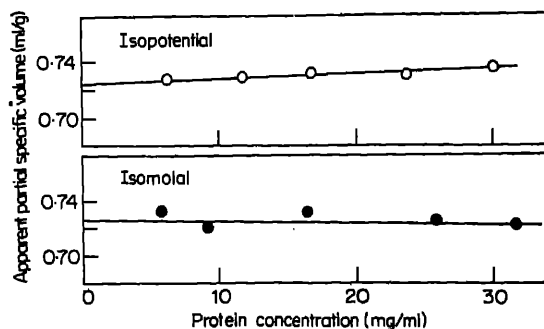
Figure 1. Concentration dependence of the apparent partial specific volume of α-globulin under isomolal and isopotential conditions in 0.1 M phosphate buffer pH 6.5 contaning 1 M NaCl.

concentration was 0.725 ± 0.002 indicating almost no preferential interaction of the protein with the solvent components to cause any distinguishable hydrodynamic change in the protein. However, it could very well be a compensatory effect of the hydration as well as that of buffer salts and NaCl. Since in an isopotential experiment, the chemical equilibrium is established, the value is taken as the partial specific volume of the protein in native condition and is listed in table 1.

Table 1. Partial specific volumes and preferential interaction parameters of α-globulin in urea and GuHCl solutions.

	Solvent	
	8 M Urea	6 M GuHCl
$\overline{V}_2^\circ$ (native)	0.725 ± 0.002	0.725 ± 0.002
$\phi_2, m_3 - \phi_{m_3} \ C \to O^a$	0.708 ± 0.004	0.707 ± 0.003
$\phi_2, m_3' - \phi_{m3} \ C \to O^c$	0.692 ± 0.002	0.684 ± 0.002
ξ_3 (from eq. 5)^b	0.08 ± 0.02	0.10 ± 0.02
$\partial m_3 \partial m_2)^c_{\mu_1, \mu_3}$	133 ± 40	105 ± 20

^{a} Values given in ml/g; ^{b} Values given in g/g;

^{c} Values given in mol/mol.

(ii) 8 M Urea buffer

The concentration dependence of apparent partial specific volumes is presented in figure 2 for α-globulin in 8 M urea buffer. At constant molality of urea a value of 0.708 ± 0.004 is obtained at zero protein concentration and at constant chemical
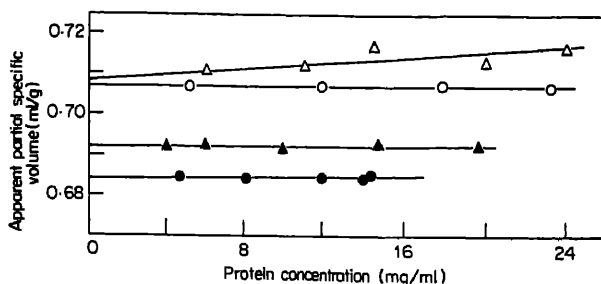
Figure 2. Concentration dependence of the apparent partial specific volume of α-globulin in 8 M urea buffer and 6 M GuHCl solutions under isomolal and isopotential conditions. Urea: Isomolal (Δ) and isopotential (▲); GuHCl: Isomolal (O) and isopotential (●).

potential a value of 0.692 ± 0.002 is obtained at zero protein concentration as shown in table 1. The results indicate a preferential interaction of solvent component with α-globulin leading to a decrease in the partial specific volume of the protein and the protein concentration has not much effect on the partial specific volume of the protein in isopotential condition and has a significant effect under isomolal conditions.

(iii) 6 M GuHCl

Figure 2 shows the partial specific volume of α-globulin in 6 M GuHCl solution at various protein concentrations. Under isomolal conditions a value of 0.707 ± 0.003 at zero protein concentration is obtained for α-globulin and the value is fairly independent of protein concentration. On the other hand, under constant chemical potential an interpolated value of 0.684 ± 0.002 is obtained at zero protein

concentration and the partial specific volume of the protein is dependent on the protein concentration. The results indicate a preferential interaction of solvent components with α-globulin leading to a decrease in the partial specific volume of the protein.

Also it can be seen from table 1 that the partial specific volume in 6 M GuHCl is less than in 8 M urea solution indicating a much higher preferential interaction of the solvent components in 6 M GuHCl with the protein.

The difference between the partial specific volume of a protein in the native and unfolded states under conditions of constant molality reflects mainly the volume change which accompanies denaturation. On the other hand, the difference between partial specific volume of the native and unfolded protein after dialysis should reflect a combination of two factors: (i) the volume change due to denaturation and (ii) preferential interaction with solvent components. From table 1, it is difficult to distinguish between the contribution from volume change upon denaturation and from preferential interaction with solvent components. In 8 M urea buffer, the volume change observed in the case of proteins such as β-lactoglobulin, papain and α-chymotrypsin is due mainly to denaturation (Lee and Timasheff, 1974a). On the other hand in the case of lysozyme, α-lactalbumin and chymotrypsinogen A, the preferential interaction parameter makes a more significant contribution to volume change (Prakash *et al.*, 1981b).

Data on partial specific volume of proteins in urea solution is scarce in the literature (Prakash *et al.*, 1981b). Most of the reported values for denatured proteins were obtained in 6 M GuHCl solution. Upon comparison of the partial specific volume of α-globulin in urea and GuHCl, it would seem that ϕ_2, m_3 values in 8 M urea are somewhat higher than those in 6 M GuHCl. This may reflect either the known fact that 8 M urea is less effective denaturing agent than 6 M GuHCl (Aune and Tanford, 1967; Salahuddin and Tanford, 1970; Tanford, 1968; 1970; Green and Pace, 1974) or simply   a difference in the electrostriction in the presence of ionic (GuHCl) and nonionic (urea) denaturants. Prakash and Nandi, (1977b) have reported the viscosity of α-globulin in both 6 M GuHCl and 8 M urea solution in triethanolamine buffer, 0.05 M, pH 9.0 containing 0.5 M KCl. In 8 M urea solution, they obtain a value of 0.28 dl/gm and under the same conditions they obtain a value of nearly 0.4 dl/gm in 6 M GuHCl solution, indicating that α-globulin is unfolded to a greater extent in 6 M GuHCl than in 8 M urea solution. This probably indicates that just as in many other proteins urea is a much less effective denaturing agent than GuHCl for α-globulin   and probably electrostriction difference between the two denaturants is not playing a major role.
Protein solvent interactions: The preferential interaction parameter for α-globulin in both 8 M urea and 6 M GuHCl was calculated according to equation 5, from the partial specific values. The volues of ξ_3 and $\partial m_3/\partial m_2$ are listed in table 1. In these calculations, the values of $\overline{V}_3$ and ρ_0 used were: for 8 M urea, $\overline{V}_3 = 0.763$ ml/g and $\rho_0 = 1.1153$ g/ml (Prakash *et al.*, 1981b) and for 6 M GuHCl, $\overline{V}_3$ ml/g (Reisler and Eisenberg, 1969) and $\rho_0 = 1.1427$ g/ml (Lee and Timasheff, 1974a). The value of $\overline{V}_3$ was determined by the same methods as those employed for the proteins with measurements done at molarities higher than 8 M urea or 6 M GuHCl followed by

extrapolation back to 8 M urea (Prakash *et al.*, 1981b) and 6 M GuHCl (Lee and Timasheff, 1974a).

In general, the value ξ_3 varies between zero (ribonuclease A) and 0.14 (pepsinogen) g of urea/g of protein (Prakash *et al.*, 1981b); and zero (ribonuclease A) and 0.17 (α-chymotrypsin) g of GuHCl/g of protein (Lee and Timasheff, 1974a). It was observed that in the well-worked systems of urea and GuHCl, the interaction was not preferential with water (Prakash *et al.*,1981b; Lee and Timasheff, 1974a). The values of ξ_3 for α-globulin also indicate that the interaction preferential with solvent components and not with water. From table 1, one can see that the preferential interaction parameter is much higher in GuHCl (0.10 g/g) than in urea (0.08 g/g) solution. It is known that most proteins are devoid of specific structural features in 6 M GuHCl (Tanford, 1968, 1970). This conclusion may be extended to the 8 M urea system although the extent of randomization of structure may be smaller in that solvent and the kinetics of denaturation may be slow in 8 M urea (Aune and Tanford, 1967; Salahuddin and Tanford, 1970; Tanford, 1968 and 1970; Green and Pace, 1974 and Pace, 1975). It is shown by Lee and Timasheff (1974a) and Prakash *et al.* (1981b) that the preferential interaction parameter ξ_3 in either 6 M GuHCl or 8 M urea, is related to the intrinsic properties of the protein molecule, especially the amino acid composition. This is more so since it is known that the different amino acid side chains differ from each other in their affinities of water, urea and GuHCl (Nozaki and Tanford, 1970).

Since preferential interaction parameter varies from one protein to another, it seemed reasonable, just as in the case of 6 M GuHCl (Les and Timasheff, 1974a) or 8 M urea (Prakash *et al.*, 1981b) to normalize the data in terms of the actual apparent binding of urea to each protein. This can be calculated from the preferential interaction parameter if the total degree of hydration is known from equation 10. A close examination of this equation reveals that a proper choice of the parameter A_1 is critical, since a range of values for A_1 of proteins can be found in the literature (Kuntz, 1971; Bull and Breese, 1968; Squire and Himmel, 1979) and also A_1 depends on the technique used (Kutz, 1971; Bull and Breese, 1968; Tanford, 1961; Timasheff, 1963). In the absence of experimental value of A_1 for α-globulin it was calculated from the hydration of its constituent amino acids according to the method of Kuntz (1971). The above procedure assumes that (i) the majority of the residues are exposed to and are in contact with the bulk solvent in the presence of 8 M urea or 6 M GuHCl and (ii) the hydration values for the proteins investigated are equal to the summation of those of their constituent amino acids. This approach appears to have a greater validity than that of taking A_1 values measured by different experimental techniques and in different solvents.

However, the values of hydration of the amino acids depends on the pH of the system also. This is exemplified in the data of Kuntz (1971) where aspartate, glutamate and tyrosyl residues show a strong pH dependence of their hydration. Since in the present study the protein α-globulin, just like other major seed proteins is rich in aspartic and glutamic acids proper hydration values have to be taken into account both in the urea system (where pH = 4) and in the GuHCl system. Values of 2 for both aspartic and glutamic acids at pH 4 and of 6 and 7.5 at

pH 7 are used for the hydration of aspartic and glutamic acids (Kuntz, 1971). The value of g_3 used in the calculation of A_3 was 0.752 g of urea/g of water and 1.007 g GuHCl/g of water, as determined gravimetrically. Incorporation of these values of A_1, g_3 and $(\partial g_3 / \partial g_2)_{T,P,\mu_3}$ (all in g/g) into equation (7) gives the value of A_3 for α-globulin. The results are summarised in table 2.

Table 2. α-Globulin-solvent interactions in urea and GuHCl solutions.

Solvent	$(\delta g_3/\delta g_2)$ T, p, μ_3 (g/g)	A_1 (g/g)	A_3		A_3 Calcd (g/g)
			(g/g)	(mol/mol)	
8 M Urea	0.08	0.252	0.270	449	0.285
6 M GuHCl	0.10	0.401	0.480	507	0.454

The interaction of urea and GuHCl with α-globulin may occur at a variety of sites. The mechanism of denaturation has been generally examined from three points of view: (i) the interaction of urea or GuHCl with a variety of sites on the protein (ii) the effect of urea or GuHCl on the solvent structure itself and (iii) a combination of these two factors. Due to the complex nature of the solvent itself and since no precise information is available on the equilibrium structure of water around various groups of a macromolecule, it is difficult to interpret the results from the point of view of solvent structure. On the other hand the favourable free energy of transfer of hydrophobic side chains and in particular of aromatic residues from aqueous medium to 6 M GuHCl or 8 M urea suggests these are likely sites of interaction (Tanford, 1970; Nozaki and Tanford, 1970). However, the hydrogen bonding ability of the guanidinium group in urea molecule (bifunctional reagent) should favour their interaction with peptide bonds. On this basis Rohinson and Jencks (1965) have postulated that GuHCl/urea molecule could hydrogen bond to two peptide bonds forming a cyclic structure. Based on this Lee and Timasheff (1974a, b) and Prakash *et al.*, (1981a, b) have proposed models for the interaction of GuHCl and urea respectively with the various proteins. From these models, the theoretical values of A_3, the value of absolute solvation, is calculated for α-globulin both in 8 M urea and 6 M GuHCl and is listed in table 2 last column. It can be seen from table 2 that in urea the experimentally determined A_3 is in excellent agreement with the theoretically calculated value of A_3. On the other hand in GuHCl even though there is a good correlation the experimentally determined A_3 has a slightly higher value.

Volume change: Using the data in table 1 and equation 6, the changes in volume upon tansfer of α-globulin from dilute salt solution to 8 M urea or 6 M GuHCl were calculated from a knowledge of the apparent partial specific volumes measured at constant molality of urea/GuHCl and under native conditions. A value of −4700 ml/mol. and −5000 ml/mol were obtained upon transferring α-globulin from dilute salt solution to 8 M urea and 6 M GuHCl solutions respectively. These values are in good agreement with the viscosity data of Prakash and Nandi (1977b)

where it is shown that α-globulin is more extended in GuHCl solution than in urea solution. The magnitude of the values appears to be comparable to many other proteins of similar nature, although not much work has been done in measuring ΔV of multimeric proteins of very high molecular weight. Also, in α-globulin, all the difference in ΔV cannot be attributed to unfolding only since Katz and Miller (1971) and Krauz and Kauzmann (1965) have shown that the ΔV values obtained at different pH's cannot be interpreted directly unless the contribution of protonation to ΔV is accounted for. From the ΔV values are also calculated ∂V in ml/mol and are shown in the last column of table 3. From a comparison of the ∂V values of many other proteins in urea and GuHCl (Lee and Timaseff, 1974a;

Table 3 Change in volume for α-globulin upon transferring from native to the denatured state in 8 M urea and 6 M GuHCl.

Solvent	$-\Delta V$ (ml/mol)	$-\partial V = \Delta V$/residue (ml/mol of residue)
8 M Urea	4700 ± 1600	2.35 ± 0.8
6 M GuHCl	5000 ± 1400	2.50 ± 0.7

Prakash *et al.*, 1981b), it appears that the value is close to that of β–lactoglobulin in 6 M GuHCl and papain in 8 M urea solution. However, no quantitative comparison can be made since various denaturants could interact differently with different proteins and such interactions need not unfold the proteins to the same final thermodynamically stable form. Even assuming it does so unless the equilibrium strucutre of water around the various groups of the macromolecule is known precisely it is difficult to interpret all the observed results of denaturation of proteins from a thermodynamic and mechanistic point of view.

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Role of S gene product of bacteriophage lambda in host cell lysis

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Abstract. Studies with the induced lysogens of λS^+R^+, λS^-R^+, λS^+R^- and λS^-R^- phages have shown that while the S gene product is essential for the action of intracellular R gene product to release the periplasmic alkaline phosphatase in the presence of EDTA, the latter gene product can bring about this effect while acting on *Escherichia coli* cells from outside, in the absence of functional S gene product; chloroform, could help the intracellular R gene product in effecting bacterial lysis in the absence of S gene product. These result support the premise that the S gene product facilitates the R gene product in crossing the cytoplasmic membrane into the periplasmic space such that the latter can act on the peptidoglycan layer of the host cell thus causing both the release of alkaline phosphatase and cell lysis.

Keywords. Bacteriophage λ; λS and R genes; periplasmic leakage; host cell lysis.

Introduction

The lytic growth of λ in *Escherichia coli* is terminated by lysis of the bacterial cell brought about by joint action of the products of three genes, R (Campbell, 1961), S (Harris *et al.*, 1967) and Rz^- (Young *et al.*, 1979), of the phage. While the induced R^- and S^- lysogens are not lysed (Reader and Siminovitch, 1971b), induced Rz^- lysogen is lysed if Mg^{2+} is absent in the medium (Young *et al.*, 1979). The R gene product is a transglycosylase which degrades the peptidoglycan layer by attacking glycosidic linkages (Bienkowska-Szewczyk and Taylor, 1980), and the Rz^- gene product has been suspected to act on the outer membrane to bring about lysis of the host cell (Young *et al.*, 1979). Comparative studies made with S^+ and S^- lysogens (Reader and Siminovitch, 1971a,b; Adhya *et al.*, 1971; Mukherjee and Mandal, 1976) have shown that the respiratory uptake of oxygen, total DNA synthesis, transport function, β-galactosidase induction are all reduced after induction in the former lysogen relative to those in the latter; the induced S^- lysogen also continues to accumulate intracellular phage beyond 60 min while the former does not. In the induced S^+ lysogen, there is evidence for phospholipid

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hydrolysis (Reader and Siminovitch, 1971b) which is considered to be secondary to the action of S gene product. Based on these observations, it has been suggested. that the target of S gene product action is the cytoplasmic membrane, and that by this action, the membrane is altered in such a way that the R protein is now able to act on its substrate *viz.* the peptidoglycan layer in the cell wall (Reader and Siminovitch, 1971b). In this paper, we present evidence to show that in the absence of the S gene product, the R gene product does not reach the peptidoglycan layer to exhibit its lytic action.

Materials and methods

Media and solutions

Tryptone broth contained 1% bactotryptone and 0.5% NaCl. Normal saline contained 0.85% NaCl. ST buffer contained 0.5 M sucrose and 0.033 M Tris-HCl, pH 8.0.

Bacteria and bacteriophage strains

Escherichia coli 594 (Sm^r, su^-, lac^-, gal^-, F^-) was obtained from Dr M. Lieb, University of Southern California, School of Medicine, Los Angeles, USA. *E. Coli* 594-APC-12, an alkaline phosphatase constitutive strain, was prepared by mutagenizing 594 with ethyl methane sulphonate according to the procedure of Lin *et al.* (1960) and selecting the mutant by the procedure of Torriani *et al.* (1961). Phage strains $\lambda cI857$, $\lambda cI857Ssus7$, $\lambda cI857Rsus5$ and $\lambda cI857Ssus7Rsus5$ were obtained from Dr S. Adhya, National Institute of Health, Bethesda USA. These phage strains will be referred to in the text as $\lambda cItsS^+R^+$, $\lambda cItsS^-R^+$, $\lambda cItsS^+R^-$, and $\lambda cItsS^-R^-$ respectively.

Growth of bacteria

Bacteria were routinely grown in tryptone broth at 30°C on a shaker, and the growth was monitored turbidimetrically at 590 nm in a spectrophotometer.

Heat-induction of lysogens

Lysogens, grown at 30°C to about 0.3 $A_{590\,nm}$, were induced at 43°C for 15 min and then incubated at 37°C for the desired period on a shaking water bath.

Assay of alkaline phosphatase

Alkaline phosphatase was assayed by the method of Garen and Levinthal (1960).

Unless otherwise stated, all the operations involved in the processing and different treatments of bacteria as described below were done at 0–4°C.

Measurement of the release of alkaline phosphatase by EDTA treatment

The bacteria were harvested, washed once with normal saline, and then suspended in ST-buffer to give an $A_{590\,nm}$ of 4.0. To this suspension, EDTA was added to a final concentration of 0.5 mM, and the mixture was divided into two equal parts. One part was kept at 4°C for 15 min with occasional shaking and then centrifuged at 10,000 g for 10 min. The supernatant was called EDTA extract. To the other part,

lysozyme was added to a final concentration of 400 µg/ml, and the mixture was incubated at 20-25°C for 40 min and then centrifuged at 10,000 *g* for 10 min. The supernatant was called lysozyme extract. The total periplasmic alkaline phosphatase was determined in the lysozyme extract and the EDTA-released enzyme, in the EDTA extract.

Measurement of alkaline phosphatase releasing activity

Preparation of substrate bacteria: E. coli 594-APC-12 was grown to about 0.6 $A_{590 \, nm}$ and processed upto the EDTA-step as described above. The EDTA treated cells were then centrifuged at 10,000 *g* for 10 min, and the pellet was suspended in ST buffer to give an $A_{590 \, nm}$ of 4.0.

Preparation of crude extract: Lysogens were grown and induced as described. After 60 min of post-induction growth, cultures were chilled; cells were harvested and suspended in 0.1 volume of 0.01 M Tris-HCl buffer, pH 8.0. The cells were then broken by sonication in a Braunsonic 1510 Sonicator. The sonicates were centrifuged at 3000 *g* for 10 min. the supernatants were dialyzed against the above buffer overnight, and the alkaline phosphatase releasing activity was then measured as follows.

To 1 ml portions of the substrate-cell suspension prepared as above were added different aliquots of the dialyzed extracts, and incubation was carried out at 4°C for 10 min. These were then immediately centrifuged at 0-4°C. Alkaline phosphatase was then measured in the supernatants. Alkaline phosphatase releasing activity was calculated from the linear region of the activity *vs* protein concentration curve.

Results

In gram negative bacteria like *E. coli,* the alkaline phosphatase and a few other hydrolytic enzymes are localized in the periplasm, the space in between the cytoplasmic membrane and the peptidoglycan layer (Heppel, 1971). The peptidoglycan layer is the most rigid layer which together with the outer membrane maintains cell shape and osmotic stability (Henning and Schwarz, 1973) and acts as a diffusion barrier (Leive, 1974). The periplasmic enzymes, under normal conditions, are not released into the medium unless the peptidoglycan layer is degraded by lysozyme added externally to EDTA-treated bacteria (Neu and Heppel, 1965); these enzymes are also released when EDTA-treated bacteria are given osmotic shock (Leive, 1965), and or when penicillin-induced spheroplasts of *E. coli* are treated with EDTA (Ananthaswamy, 1977).

Based on the release of periplasmic enzymes as a consequence of the structural damage of the peptidoglycan layer as mentioned above, we thought that the action of intracellular *R* gene product of λ on this layer could be monitored by measuring the release of periplasmic enzymes from the induced lysogen under suitable conditions. While working with a converted polylysogen of λ, we observed that this bacterium, unlike its nonlysogenic parent, released periplasmic enzymes on treatment with 0.1 mM EDTA in hypertonic medium (Mandal and Barik, 1979; Barik, 1981). So, to determine whether the availability of intracellular *R* gene

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product for its action on the peptidoglycan layer is dependent on the functional S gene product, we tested the ability of different lysogens of λ collected at different times after induction to release periplasmic alkaline phosphatase on treatment with EDTA. The experiment was done as described under legend to figure 1. It can be
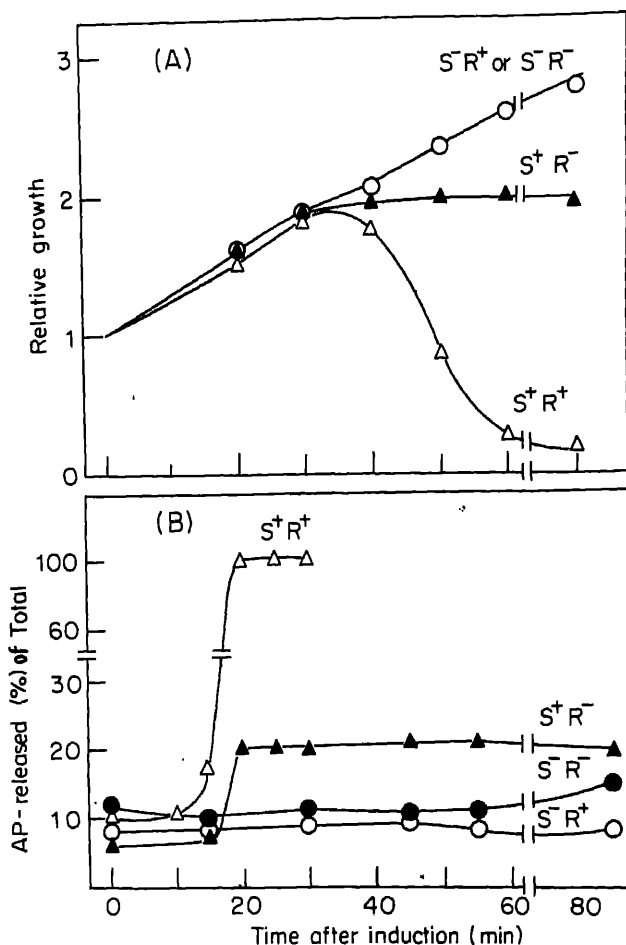
Figure 1. Growth of different lysogens after induction and the release of alkaline phosphatase by EDTA treatment of induced λ lysogens. Lysogens of $\lambda cIts S^+R^+$, $\lambda cIts S^-R^+$, $\lambda cIts S^+R^-$ and $\lambda cIts S^-R^-$ in *E. Coli 594*-APC-12 were grown and induced as described in Methods. During post-induction growth, aliquots were taken out at indicated times and chilled. A portion from each aliquot was used to measure the growth and the residual portions were used to measure the release of alkaline phosphatase by EDTA treatment as described in Methods. A: Growth; the relative growth was measured by taking the ratio of $A_{590\,nm}$ of the induced culture at indicated time to that at zero time; the $A_{590\,nm}$ at zero time varied from 0.2 to 0.3 for different cultures (1 $A_{590\,nm} = 5 \times 10^8$ cells/ml during log phase of growth for this strain). B: Release of alkaline phosphatase. Other details are in methods.

seen from figure 1B that the S^+R^+ lysogen started showing increased EDTA-induced periplasmic leakiness above the basal level at 15 min which reached 100% at 20 min from the start of induction. On the other hand, the induced S^-R^+

and S^-R^- lysogens did not show any such increase in this leakiness even upto 60 min of post-induction growth. In contrast with the latter two lysogens, induced S^+R^- lysogens showed a slight increase in the leakage which reached 20% at about 20 min and remained unchanged thereafter. An examination of the growth pattern (figure 1A) reveals that the induced S^+R^+ lysogen started lysing after about 35 min, while S^-R^+ and S^-R^- lysogens, under identical conditions, showed a steady increase in the turbidity beyond that period; but the induced S^+R^- lysogens neither lysed nor showed any increase in turbidity after 35 min.

To ensure that the alkaline phosphatase releasing activity was associated with the R gene product of λ and that this was present inside the induced S^-R^+ lysogen, the above activity was measured in the crude extracts prepared from different lysogens harvested at 60 min after induction. The results presented in table 1 show that the amount of alkaline phosphatase releasing activity was maximum in the extract of induced S^-R^+ lysogen; the extracts of induced S^+R^- and S^-R^- lysogens contained respectively 1.4% and 0.1% of the activity present in the S^-R^+ extract.

Table 1. Alkaline phosphatase releasing activity at 60 min after induction of different lysogens.

Lysogen	Alkaline phosphatase releasing activity[a]	
	(units/mg protein)[b]	%
594 (λcItsS⁻R⁻)	2540.0	100.0
594 (λcItsS⁺R⁻)	35.00	1.4
594 (λcItsS⁻R⁻)	2.5	0.1

[a] Details are given in Methods.

[b] The unit of alkaline phosphatase releasing activity was defined as the amount of protein which released one unit of the enzyme from the substrate cells during 10 min of incubation at 4°. One unit of alkaline phosphatase was defined as the amount of enzyme which could liberate 1.0 absorption unit of p-nitrophenol (measured at 420 nm) from p-nitrophenyl phosphate under the condition of assay in 10 min. The extract from a noninduced lysogen was used in blank experiment.

These results (figure 1 and table 1) suggest the following: (i) The R product-independent alkaline phosphatase releasing activity in these lysogens is very negligible compared to the R dependent one; (ii) though the R product dependent alkaline phosphatasee releasing activity was present inside the induced S^-R^- lysogen at a very high concentration (table 1), it could not act on the peptidoglycan layer in the absence of the functional S gene product (figure 1); (iii) the same R product present in the extract of S^-R^- lysogen could act from outside on the peptidoglycan layer of EDTA treated bacteria effecting the release of periplasmic alkaline phosphatase in the absence of S gene product.

The failure of intracellular R product to act on the peptidoglycan layer in induced S^-R^+ lysogen (figure 1) suggests, therefore that the former could not reach its substrate which is situated outside the cytoplasmic membrane. So, the cytoplsamic membrane seems responsible for the above non accessibility of the intracellular R product to the peptidoglycan layer in the absence of S product. The cytoplasmic membrane has the lipid bilayer structure (Davson and Danielli, 1943), and any lipid solvent can disrupt this bilayer structure by solubilizing the lipid components (Dewey and Barr, 1970). If the role of S gene product in host cell lysis is to damage the cytoplasmic membrane through which the R product can move out into the periplasm to become accessible to the peptidoglycan layer, then chloroform, a lipid solvent, can be expected to bring about such a phenomenon in the absence of S protein. The results of the experiment presented in figure 2 clearly
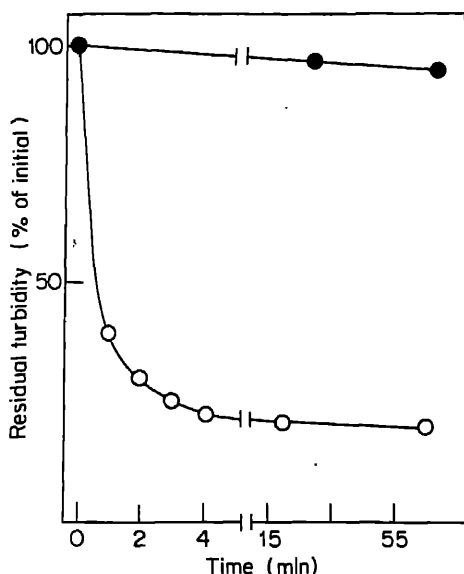
Figure 2. Effect of chloroform on nonlysogen and induced lysogens of different λ phages. Lysogens were grown and induced as described in methods. At 60 min after induction, cultures were chilled, and cells were harvested and then suspended in 0.05 M potassium phosphate buffer, pH 7.4 at a cell density of 3×10^9/ml. This suspension was then diluted 10-fold in the same buffer previously saturated with chloroform, and the decrease in turbidity with time was followed spectrophotometrically at 590 nm. O, S^-R^+ lysogen; ●, S^-R^- or S^+R^- lysogen or 594.

show that in the presence of chloroform, induced S^-R^+ cells were lysed rapidly while S^-R^- and S^+R^- cells were not. These results indicate that in the presence of chloroform, the R product is accessible to the peptidoglycan layer to exert its lytic action even in the absence of the S gene product. It may also be noted that chloroform, by itself, cannot effect lysis unless the functional R gene product is present inside the cell.

Discussion

As the peptidoglycan layer is structurally the most rigid component of the bacterial envelope, its degradation is a must for lysis of the cell. In order that the R gene

product of λ could reach this rigid layer while approaching from inside, it has to cross the cytoplasmic membrane.

We have shown that the intracellular action of R gene product on the peptidoglycan layer can be monitored by measuring the EDTA-induced release of periplasmic alkaline phosphatase from intact bacterial cells. It has been shown further that the intracellular R product cannot effect release of any alkaline phosphatase unless functional S gene product is also present (figure 1), though the former can bring about such a release while acting from outside, even in the absence of the latter (table 1). This directly supports the premise that the S gene product helps the R gene product in reaching the periplasmic space to act on the peptidoglycan layer.

With regard to the mechanism by which the S product helps the R product in crossing the cytoplasmic membrane nothing is known; from the known effects of S gene product on the properties and functions of the cytoplasmic membrane discussed earlier (see introduction), it appears possible that this protein, would somehow derange the structure of the above layer thus allowing the cytoplasmic R protein to enter the periplasm; this premise is supported by the fact that chloroform, by its lipid solubilising action on the membrane, performs the function of S gene in facilitating R gene product to become accessible to the peptidoglycan layer (figure 2).

In the absence of the R gene product, the S gene product also contributes towards the EDTA-induced leakage of alkaline phosphatase (figure 1 and table 1). The possibility that this is not due to the primary effect of S gene product is evident from the fact that after induction of λ lysogen, the intracellular level of S gene product along with those of several other late proteins of the phage increases with time (Reader and Siminovitch, 1971a), and this would have caused a progressive increase in the release of alkaline phosphatase provided that this protein bear any direct relationship with the release phenomenon. But the results in figure 1 show that this release from induced S^+R^- lysogen reaches 20% at 20 min and remains unchanged thereafter. It is, therefore conceivable that the S protein dependent small release is secondary to the action of this protein on the cytoplasmic membrane. When the S protein damages or deranges the structure of cytoplasmic membrane, the membrane-bound lytic enzymes of bacterial origin (Daneo-Moore and Shockman, 1977) may be released in a soluble form and these can be expected to act either from inside (figure 1) or from outside (table 1), on the peptidoglycan layer thereby causing a small relase of alkaline phosphatase.

The mechanism of S protein action on the cytoplasmic membrane is not yet clear. It is however known that when the multiplication of the phage having *ts* mutation in one of its replication genes is arrested by continuous growth at 44°C, the cells which are not lysed even after 2 h, are now lysed on addition of chloroform (Mandal, unpublished result). Also, it was shown by others that the initiation of lysis is not solely determined by the intracellular level of R gene product but must await the intraceullular accumulation of S gene product (del Campillo-Campbell and Campbell, 1965; Protass and Korn, 1966). These observations suggest that the action of the above gene product is not catalytic unlike that of R gene product. The

stoichiometric mechanism of action of the S protein was also suggested by Reader and Siminovitch (1971a). Further studies are necessary to unravel the exact mechanism of its action on the cytoplasmic membrane.

Acknowledgements

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Production of antibodies specific to human chorionic gonadotropin in mice immunized against its chemical analogs†

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Abstract. Mice immunized against DS_5-hCG-β and DS_6-hCG-β, chemical analogs of β-subunit of human choriogonadotropin (hCG-β) in which 5 and 6 disulphide bonds respectively were reduced and alkylated, were found to produce antibodies specific to hCG without significant crossreactivity with human lutropin (hLH) as tested in a radioimmunoassay. In contrast, mice immunized against the native hCG-β subunit produced hLH crossreacting antibodies. While the anti-DS_5, DS_6-hCG-β serum was capable of selectively blocking the binding of $[^{125}I]$-hCG to rat testicular LH/hCG receptors, it failed to inhibit the binding of $[^{125}I]$-hLH to the same receptors. The radioimmunoassay for hCG using the mouse anti-DS_5, DS_6-hCG-β serum was not as sensitive as that employing rabbit anti-DS_5, DS_6-hCG-β serum. The minimal detection limit was 5 ng/ml for the mouse antibody as compared to 1 ng/ml for the rabbit antibody.

Keywords. hcG Specific antibody; disulphide modified hCG-β-subunit; immuno-contraceptive.

Introduction

Although antibodies to human choriogonadotropin β-subunit (hCG-β) can discriminate between hCG and human lutropin (hLH), they lack absolute specificity. The degree of hLH crossreactivity of the antibody varies with the preparation of hCG-β, ranging from 10 to 30%. Nevertheless, antibody to hCG-β is considerably more specific than antibody to hCG and this specificity is most likely due to the differences in the physicochemical characteristics of hLH and hCG-β (Bhal, 1980). hCG-β not only has an additional 30-amino acid residue sequence at its carboxy-terminus but also has certain other domains in the polypeptide chain with variable sequences. Furthermore hCG-β undergoes a

Abbreviations used: hCG, human choriogondotropin; hCG-α and hCG-β, α and β subunits of hCG; hLH, human lutropin; hFSH, human follitropin; hTSH, human thyrotropin; hPRL, human prolactin; DS_5hCG-β-hem and DS_6hCG-β-hem, reduced and S-alkylated hCG-β in which 5 and 6 disulphide bonds were modified respectively and then coupled with hemocynanin; RIA, radioimmunoassay; CMC, carboxymethyl cysteine; TC buffer, 0.05 M Tris-HCl buffer pH 7.2 containing 1 mM $CaCl_2$.

conformational change when it is dissociated from hCG-α (Hilgenfeldt *et al.*, 1974). The antigenic determinants being conformational in nature, this change in the conformation results in the loss of hLH crossreactivity of the sites. We have attempted to modify intact hCG-β in order to enhance its specificity as an antigen rather than use the unique carboxyterminal peptide which is a weak antigen (Matsuura *et al.*, 1979). Earlier work from our laboratory has demonstrated that progressive reduction and alkylation of disulphide linkages in hCG-β results in loss of antigenic determinants common to hCG and hLH (Bahl *et al.*, 1976). Among the various derivatives, reduced and S-alkylated hCG-β in which 5-disulphide bonds were modified (DS$_5$-hCG-β) retained only determinants unique to hCG with complete loss of those crossreacting with hLH antibodies (Ghai *et al.*, 1980). Immunization of rabbits with DS$_5$-hCG-β prepared according to the methods standardized in our laboratory yielded antibodies specific to hCG by immunological and biological criteria (Pandian *et al.*, 1980). With the idea that this chemical derivative of hCG-β would be useful as a means of immunological control of fertility in humans, studies were initiated to test the immunogenic specificity of the antigen in other animals. We report here that a sequential injection of DS$_5$-hCG-β and DS$_6$-hCG-β, a fully reduced and alkylated derivative of hCG-β, into mice resulted in antibodies specific to hCG. As a control, another group of mice was immunized against native hCG-β. This group of mice produced antibodies to hCG which were crossreactive with hLH to a significant extent.

Materials and methods

Sephadex-G-25 (coarse), G-100, Sepharose-4B and diethylaminoethyl (DEAE) Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, USA. Freund's complete adjuvant was a product of Gibco, Grand Island, New York, USA. Carrier free Na^{126}I was purchased from Amersham Corp., Arlington, Illinois, USA. Bovine-γ-globulin, bovine serum albumin and glutaraldehyde (25% solution) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals were of certified Analytical grade. BALB/C mice were used in this study.

Preparation of DS$_5$-hCG-β and DS$_6$-hCG-β

The β-subunit of hCG was prepared by dissociation of hCG in 8M urea and chromatography over DEAE-Sephadex-A 50 (Swaminathan and Bahl, 1975). To remove the last traces of hCG, the β-subunit was treated with an immunoadsorbent prepared by coupling antibodies to hCG-α to CNBr-activated Sepharose (Pandian *et al.*, 1980). After determining the binding capacity of the immunoadsorbent hCG-β was treated with a calculated amount of the immunoadsorbent. Details of the procedure are given elsewhere (Pandian *et al.*, 1980). The anti hCG-α immunoadsorbent treated hCG-β was then partially or completely reduced and alkylated to prepare DS$_5$-hCG-β and DS$_6$-hCG-β respectively (Ghai *et al.*, 1980). Amino acid analysis for carboxymethylcysteine residues was later carried out to confirm the degree of reduction. The hLH cross-reactivity of the preparation was monitored by radioimmunoassay of DS$_5$-hCG-β in a [^{125}I]-hLH-anti-hLH radio immunoassay (RIA) system. In order to ensure the complete removal of hLH

crossreactivity of the antigen, it was treated with a calculated amount of an immunoadsorbent, prepared by coupling antibodies to hLH to CNBr-activated Sepharose. The efficacy of the treatment was monitored by a homologous hLH RIA. This material was then conjugated to Keyhole limpet hemocyanin in a 1:1 ratio (w/w) using glutaraldehyde as the bifunctional cross-linking reagent (Pandian *et al.*, 1980). The resulting conjugate, DS_5-hCG-β-hem and DS_6-hCG-β were used to immunize mice.

Immunization of mice and collection of blood samples

Mice were immunized by intraperitoneal injection. A water in oil emulsion was prepared by mixing equal volumes of a saline solution of the immunogen [hCG-β or DS_5-hCG-β coupled with hemocyanin (DS_5-hCG-β-hem) or DS_6-hCG-β] and Freund's complete adjuvant. Mice received two injections of DS_5-hCG-β-hem (250 µg/mouse) one month apart. Twenty day after the second injection they received DS_6-hCG-β (100 µg/mouse). A second group of mice was immunized similarly by these injections of native hCG-β-subunit (100 µg/mouse). Blood samples were collected by occular puncture. Serum was later separated and frozen with 0.01% sodium azide as a preservative.

Titer of antisera

Serum (200 µl) at various dilutions (1:10 to 1:10,000) was incubated with [^{125}I]-hCG for 1 h at 37°C followed by the addition of 10 µl of 10% ammonium acetate in ethanol. The tubes were kept for 1 h at 4°C, then centrifuged, supernatants decanted, residual liquid removed by wiping with filter paper strips and finally the pellets were counted for antibody bound readioactivity. The dilution of the antiserum binding 20% of the [^{125}I]-hCG (40-50 µCi/µg) was chosen for radioimmunoassay.

Radioiodination and radioimmunoassay

The radioiodination of highly purified hCG for RIA was performed essentially by the chloramine-T procedure of Greenwood *et al.* (1963) with minor modifications.

The radioimmunoassay was performed essentially as per the procedure of Bellisario and Bahl (1975). The results were expressed as $B/B_0 \times 100$ and plotted against log dose of hCG standards. B_0 represents cpm radioactivity bound in the absence of any competing antigen and B represents cpm radioactivity bound in the presence of various levels of competing standard hCG or unknown sample. The radioimmunoassay data were also analyzed by a computer, Wang Model 2200, using program RIADS-2200, based on the method developed by Rodbard and Lewald (1970).

Radioimmuno-receptor assay

Rat testicular homogenate was used as a source of hCG/hLH receptors. Testes were removed from a healthy adult rat and placed on ice. After decapsulating the pair of testes was homogenized in 20 ml of 0.05 M Tris-HCl buffer pH 7.2 containing 1 mM $CaCl_2$ (TC buffer) in a motorized Potter-Elvehjem tissue grinder

(no more than 6 strokes). The homogenate was centrifuged at 1,000 g for 15 min in a Sorvall-RC 2B refrigerated centrifuge. The pellet was resuspended in 40 ml of TC buffer containing 0.2% BSA. Antisera (200 µl) serially diluted or standards were incubated with 0.5 ml aliquots of the testicular homogenate and 100,000 cpm of [^{125}I]-hCG or [^{125}I]-hLH for 1 h at 37°C in a Dubnoff metabolic shaker. At the end of the incubation, the tubes were centrifuged at 4°C at 2000 g for 15 min, the supernatants decanted, the pellets washed with 2.5 ml of chilled TC buffer by resuspension and centrifuged at 2000 g for 15 min. The supernatants were decanted, and the liquid adhering to the walls wiped with filter paper strips. The pellets were counted for receptor bound radioactivity. Results were expressed as B/B$_0$×100 *vs* log dose of standard hormone or dilution of antiserum. B$_0$ represents radioactivity (cpm) bound to receptors in the absence of competing standard hormone or antiserum and B represents radioactivity bound to the receptors in the presence of standard hormone or antiserum. Nonspecific binding was determined by obtaining cpm bound in the presence of excess of the cold hormone (hCG or hLH).

Results and discussion

The antigen

The hCG-β subunit prepared by dissociation of highly purified hCG with urea had 0.5% contamination as found by the radioreceptor assay. When treated with an immunoadsorbent of anti hCG-α antibodies, the contamination (hCG) was completely removed (Pandian *et al.*, 1980). This hCG-β was then reduced partially under controlled conditions and alkylated to yield DS$_5$-hCG-β. On analysis, a value of 10.4 was obtained for carboxymethylcysteine residues pointing to the reduction on an average of 5.2 disulphide bridges (Ghai *et al.*, 1980). The material was tested in [^{125}I]-hLH-and-hLH RIA system to monitor the presence of hLH-cross reacting material. This amounted approximately to 0.2–0.3% (figure 1) of hLH activity. However, after treatment with an
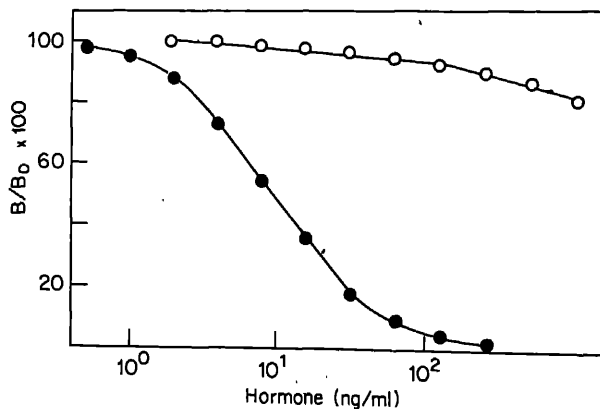
Figure 1. Radioimmunoassay of DS$_5$-hCG-β in the ^{125}I-hLH-anti-hLH system. Human LH (●) and DS$_5$-hCG- (○) were separately used as standards in an RIA using ^{125}I-hLH and anti-hLH serum (1:30,000 dilution of antiserum). For details of the assay, see the text. Results are expressed as B/B$_0$×100 vs log dose of the competing antigen. The value of B$_0$ ranged from 9,000–10,000 cpm corresponding to 27% to 30% specific binding.

immunoadsorbent of anti-hLH-Sepharose, the crossreactivity was reduced to negligible level (0.01% hLH activity). DS_5-hCG-β conjugated to Keyhole limpet hemocyanin was then dialyzed and lyophilized to yield DS_5-hCG-β-hem. DS_6-hCG-β was prepared by complete reduction and alkylation of hCG-β. Amino acid analysis gave an average value of 11.96 for the number of CMC residues indicating that 5.98 disulphide bridges had been reduced and alkylated (Ghai *et al.*, 1980).

The antisera

Mice were immunized against native hCG-β or DS_5-hCG-β-hem as described under Methods. The serum levels of antibody after the second injection are shown in Figure 2. The anti-DS_5-hCG-β-hem serum showed very minimal
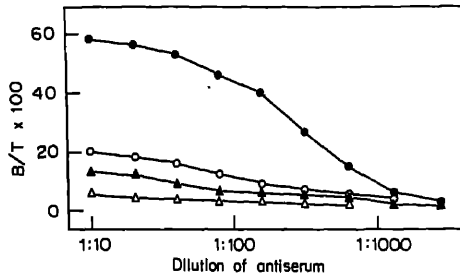
Figure 2. Titer and crossreactivity of anti-hCG-β serum and anti DS$_5$-hCG-β serum from mice immunized against the respective antigens. Data from a representative mouse is given here for each antigen. For details of procedure see the text. Results are expressed as B/T $\times$ 100 *vs* log dilution of antiserum where B represents cpm bound to the antibody and T is the total cpm added to the incubation. ($\bullet$) Binding of ^{125}I-hCG to anti-hCG-β serum; ($\blacktriangle$) Binding of ^{125}I-hLH to anti-hCG-β serum; (O) Binding of ^{125}I-hCG to anti-DS$_5$-hCG-β serum; ($\triangle$) Binding of ^{125}I-hLH to anti-DS$_5$-hCG-β serum.

binding to $[^{125}$I]-hLH. However, after receiving the third injection (with DS_6-hCG-β), the antibody level against hCG was elevated with negligible binding to $[^{125}$I]-hLH (figure 3). In comparison, the serum from mice immunized against
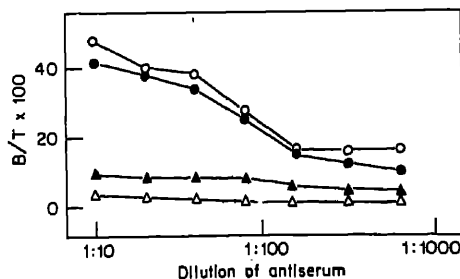
Figure 3. Titer and crossreactivity of anti-hCG-β serum and anti-DS$_5$, DS$_6$-hCG-β serum from mice immunized against the respective antigens. Data from one representative mouse is given here for each antigen. For details of procedure see the text. Results are expressed as B/T $\times$ 100 dilution of the antiserum. For explanaton, see the legend to figure 2.

native hCG-β, bound [^{125}I]-hLH to an appreciable extent. When tested in an RIA using 1:100 dilution, hLH did not interfere to any appreciable degree when anti-DS$_5$-hCG-β serum was used (figure 4). In the case of anti-hCG-β serum, however, hLH inhibited the binding of [^{125}I]-hCG to a significant degree (from 40 ng/ml and
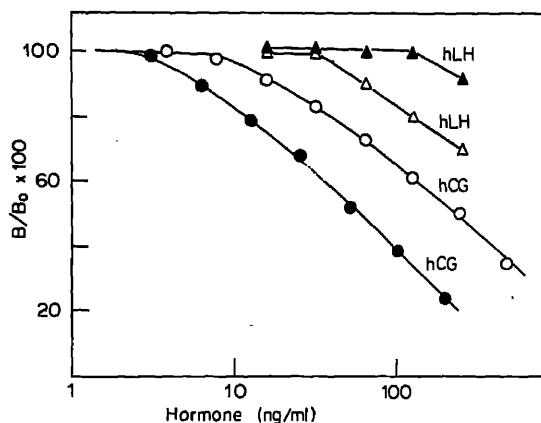
Figure 4. Radioimmunoassay of hCG and hLH using 1:100 dilution of mouse anti-hCG-β (O) and anti-DS$_5$-, DS$_6$-hCG-β sera (●). For details of the assay see the text. Results are expressed as B/B$_0$ × 100 *vs* log dose of the competing antigen where B represents cpm bound to the antiserum in the presence of graded doses of the competing antigen and B$_0$ represents cpm bound to the antiserum in the absence of any competing antigen. The value for B$_0$ was 8000 cpm representing 20% specific binding.

higher). The sensitivity (minimal detection limit) of the assay using mouse anti-DS$_5$, DS$_6$-hCG-β-hem serum was 5 ng/ml. This, however, was lower than that of the rabbit antibody based RIA which could measure 1 ng/ml with confidence (K. Muralidhar, G. Chaudhuri, J. Lippes and O. P. Bahl – unpublished data). This confirms and extends our earlier report that the rabbit antibody to DS$_5$, DS$_6$-hCG-
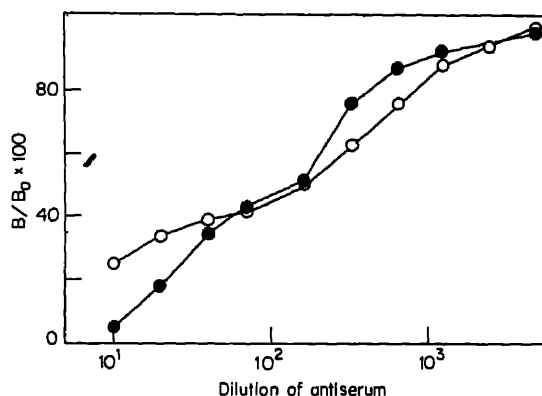
Figure 5. Radioreceptor assay of hCG in the presence of antibodies to DS$_5$, DS$_6$-hCG-β from rabbits (O) and mice (●). Rat testicular homogenate (0.5 ml) was incubated with [^{125}I]-hCG and serial dilutions of rabbit or mouse anti-DS$_5$, DS$_6$-hCG-β serum. At the end of the incubation, the receptor bound radioactivity was determined. For details see the text. Resuls are expressed as B/B$_0$ × 100 where B and B$_0$ represent cpm bound in the presence or absence of various dilutions of either rabbit or mouse antibody. The value for B$_0$ was 10,000 cpm representing 13% specific binding to receptors.

β was specific to hCG with little or no crossreaction with hLH (Pandian *et al.*, 1980). In order to ascertain whether the mouse anti-DS$_5$, DS$_6$-hCG-β serum was capable of selective neutralization of biological activity of hCG, the radio-receptor assay was carried out. As shown in figures 5 and 6 the mouse antibody was capable
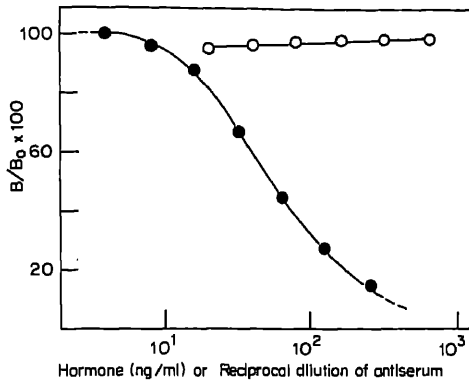
Figure 6. Radioreceptor assay of [^{125}I]-hLH in the presence of antibodies to DS$_5$, DS$_6$-hCG-β from mice. Rat testicular homogenate (0.5 ml) was incubated with [^{125}I]-hLH and serial dilutions of mouse anti-DS$_5$, DS$_6$-hCG-β serum (O) or standard hLH (●) in amounts indicated in the figure. At the end of the incubation, the receptor bound radioactivity was determined. For details see the text. Results are expressed as B/B$_0$×100 where B$_0$ represents cpm bound in the absence of any competing LH or antibody and B represents cpm bound in the presence of various dilutions of the antibody or standard hLH. The value for B$_0$ was 9000 cpm representing 10% specific binding to receptors.

of blocking the binding of [^{125}I]-hCG to testicular receptors. For comparison, the effect of rabbit antibody on the binding of [^{125}I]-hCG to receptors is also depicted in figure 5. In contrast, the binding of [^{125}I]-hLH to testicular receptors was unaffected by the presence of mouse anti-DS$_5$, DS$_6$-hCG-β serum (figure 6). This again demonstrates and confirms the biological specificity of the antibody.

A number of placental antigens have been proposed as immuno-contraceptive agents (Bhal and Muralidhar, 1980). Among these only hCG has been investigated in great detail. Active immunization studies in subhuman primates involving hCG-β have demonstrated the possibility of interrupting pregnancy with this immuno-gen (Hearn, 1979). These studies have, at the same time, indicated the existence of hLH crossreactivity in the anti-hCG-β antibodies. The degree of crossreactivity with hLH, however, varied from animal to animal. As the ideal placental immuno-contraceptive agent is envisaged as one eliciting antibodies specific to itself without cross reactivity with any non-placental tissue antigen including pituitary LH, there is a need to improve the specificity of hCG-β antibodies. Although the unique carboxy terminal peptide of hCG-β produces antibodies to hCG, some of which are capable of neutralizing the biological activity of hCG-β an action through which it is hoped to terminate pregnancy, they are not specific. Hence, an alternative approach, presented here and elsewhere (Pandian *et al.*, 1980) to use chemical derivatives of hCG-β was investigated. These studies revealed that

among the derivatives of hCG-β, DS$_5$-hCG-β and DS$_6$-hCG-β were least crossreactive with anti-hLH sera (Ghai *et al.*, 1980). It has been shown here that mice generate antibodies specific to hCG when they are immunized against DS$_5$-hCG-and DS$_6$-hCG-β. Although in a direct binding assay, the antibodies displayed binding to [^{125}I]-hLH, the binding was of low affinity as compared with the binding to [^{125}I]-hCG. This was evident from the fact that in a competitive test system such as RIA or RRA, the antibody did not show any binding to hLH. In contrast, the antibodies to hCG-β bound [^{125}I]-hLH in a direct binding assay and this binding apparently was of considerable affinity as even in an RIA the antibody binding to [^{125}I]-hCG was inhibited by the unlabeled hLH.

The immunological and biological specificity of the anti DS$_5$, DS$_6$-hCG-β sera in both rabbits (Pandian *et al.*, 1980) and mice points to the possible use of DS$_5$, DS$_6$-hCG-β as an immunocontraceptive agent. The lymphocytes from these mice immunized against DS$_5$-hCG-β and DS$_6$-hCG-β have been used to develop monoclonal antibodies to hCG without any cross reaction with hLH (Muralidhar *et al.*, 1981). The biological effects of active immunization with DS$_5$-hCG-β and DS$_6$-hCG-β are presently being investigated in a number of laboratory animals including subhuman primates.

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Dosage compensation and sex determination in *Drosophila*: mechanism of measurement of the X/A ratio +

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Abstract. We propose a molecular mechanism for the intra-cellular measurement of the ratio of the number of X chromosomes to the number of sets of autosomes, a process central to both sex determination and dosage compensation in *Drosophila melanogaster*. In addition to the two loci, *da* and *Sxl*, which have been shown by Cline (*Genetics*, **90**, 683, 1978) and others to be involved in these processes, we postulate two other loci, one autosomal (ω) and the other, X-linked (π). The product of the autosomal locus *da* stimulates ω and initiates synthesis of a limited quantity of repressor. *Sxl* and π, both of which are X-linked, compete for this repressor as well as for RNA polymerase. It is assumed that *Sxl* has lower affinity than π for repressor as well as polymerase and that the binding of polymerase to one of these sites modulates the binding affinity of the other site for the enzyme. It can be shown that as a result of these postulated interactions transcription from the *Sxl* site is proportional to the X/A ratio such that the levels of *Sxl⁺* product are low in males, high in females and intermediate in the intersexes. If, as proposed by Cline, the *Sxl⁺* product is an inhibitor of X chromosome activity, this would result in dosage compensation. The model leads to the conclusion that high levels of *Sxl⁺* product promote a female phenotype and low levels, a male phenotype. One interesting consequence of the assumptions on which the model is based is that the level of *Sxl⁺* product in the cell, when examined as a function of increasing repressor concentration, first goes up and then decreases, yielding a bell-shaped curve. This feature of the model provides an explanation for some of the remarkable interactions among mutants at the *Sxl, da* and *mle* loci and leads to several predictions. The proposed mechanism may also have relevance to certain other problems, such as size regulation during development, which seem to involve measurement of ratios at the cellular level.

Keywords X-Chromosome transcription; sex-lethal mutations; maternal effect; RNA polymerase; size regulation.

Introduction

Bridges (1925) has shown that in *Drosophila melanogaster* the sexual phenotype is determined by the ratio of the number of X chromosomes (X) to the number of sets of autosomes (A). This X/A ratio, or Bridges' ratio, also regulates the rate at

which most X-linked genes are transcribed (Lucchesi, 1973; Maroni and Plaut, 1973; Stewart and Merriam, 1978; Chandra, 1979). As a result, in flies with an integral number of chromosomes, the level of activity of enzymes coded by X-linked genes is proportional to the number of copies of the structural gene divided by the Bridges' ratio (Chandra, 1979). The end result of this regulatory process, known as *dosage compensation*, is that the phenotype resulting from two doses of a given X-linked gene in the female (AAXX) is equal to that resulting from one dose in the male (AAXY). This is the consequence of the single X chromosome in the male being transcribed at rougly twice the rate as each of the two chromosomes in the female. *D. melanogaster* is able to sustain wide variation in X/A ratios, and it has therefore been possible to show that dosage compensation operates over a variety of chromosome constitutions. Since both the sexual phenotype and dosage compensation appear to be cell-autonomous properties (Bridges, 1930; Lakhotia and Mukherjee, 1969), an intriguing feature of these two phenomena is the mechanism by which the X/A ratio is assessed within cells. Mutations which interfere with the capacity to measure the X/A ratio and, as a consequence, affect dosage compensation or sex determination, might provide insight into the molecular mechanisms involved in these processes.

Cline (1978, 1980) has made an elegant study of the following mutations which appear to fulfil such a purpose. (i) Daughterless (*da*) is a temperature-sensitive autosomal recessive (2-41.5) (Bell, 1954; Cline, 1976). Homozygous females leave behind only male offspring because the daughters die during embryonic development. Daughters can be rescued from *da/da* mothers following early injection of wild type (*da+*) egg cytoplasm (Bownes *et al.*, 1977), suggesting that the daughterless phenotype is caused by the absence of some diffusible product coded for by the *da+* locus. (ii) Sex-lethal, male-specific (*Sxl^M1*), is an X-linked mutation (1-19.2) (Cline, 1978), lethal to males and, curiously, also a dominant suppressor of *da*. (iii) Sex-lethal, female-specific, (*Sxl^F1*), is also X-lined (Muller and Zimmering, 1960; Zimmering and Muller, 1961), 0.007 recombination units away from *Sxl^M1* (Cline, 1978). It was isolated as a dominant mutation but later studies have shown that it normally behaves as a recessive and that its occasional dominant character is dependent on some undefined elements of the genetic background and on certain environmental conditions (Cline, 1978).

Cline has shown that the effects of these mutations can be explained on the following bases. (a) A maternal factor is produced by *da+*, the wild type allele of the *da* locus. In a fertilized egg whose X/A ratio corresponds to that of a female, this factor activates transcription at the *Sxl* locus. (b) The *Sxl^M1* locus is the control region of the *Sxl* gene and the *Sxl^F1* locus is the structural part. (c) The *Sxl+* product is essential for females and lethal for males. (d) The *Sxl^M1* mutation makes the synthesis of *Sxl+* product constitutive, that is, independent of stimulation by the *da+* factor. Based on these and other results, Cline has made the conjecture that the *Sxl+* product might itself be involved in dosage compensation and sex determination (Cline, 1978, 1979a). The mechanism by which X/A ratio is measured in the embryo is, however, an undefined aspect of Cline's interpretation.

In this paper we (i) present a model to show how measurement of the X/A ratio can be effected; (ii) show that the level of Sxl^+ product is proportional to the X/A ratio; and, (iii) postulate that there is a quantitative relationship between the sexual phenotype and the Sxl^+ product such that increasing cellular concentration of this product leads to increasing 'femaleness' while decreasing concentrations result in 'maleness'. The model also provides an explanation for the interactions among some of the related mutants affecting sex determination and dosage compensation.

The reasoning which led us to this model has been briefly outlined in a recent publication (Gadagkar *et al.*, 1981).

The model

Qualitative aspects

The model (figure 1) consists of five components: (i) the da^+ factor, produced in the mother and stored in the egg; (ii) a postulated autosomal site ω capable of
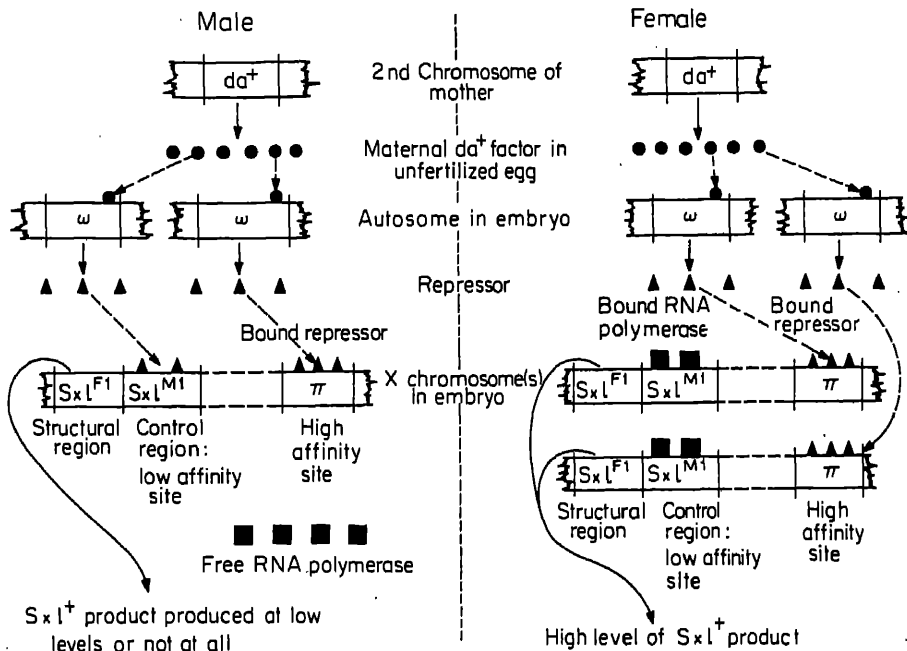
Figure 1. A model for the measurement of the ratio of the number of X chromosomes to the number of sets of autosomes.

The da^+ factor (●) is produced in excess by the maternal gene da^+ and stored in the egg. Following fertilization, this factor binds to a specific autosomal site ω in the embryo resulting in the production of a small quantity of repressor (▲). In the male embryo (left half of the figure), there is only one X chromosome and therefore only one set of low affinity Sxl and high affinity π sites. The repressor is able to bind significantly to both these sites. As a result, on the average, little or no RNA polymerase (■) binds to the Sxl site and little or no Sxl^+ product is produced. In the female embryo (right half of the figure), there are two X chromosomes but the same quantity of repressor as in the male. This quantity of repressor is just sufficient to significantly block the π sites. RNA polymerase binds to Sxl and initiates synthesis of the Sxl^+ product. Females are viable at high levels of Sxl^+ product and males at low levels.

binding da^+ factor and releasing repressor; (iii) the Sxl locus, which in our model is the *low affinity site,* capable of binding both the repressor and RNA polymerase; (iv) a postulated *high affinity site* π, also on the X chromosome, capable of binding both repressor and RNA polymerase with a much higher affinity than the Sxl locus; and (v) *RNA polymerase* which binds to both the high and low affinity sites and whose binding to the low affinity site results in transcription at the Sxl locus. RNA polymerase binds to Sxl and π with an affinity which is less than that of the repressor for these two sites. Binding of RNA polymerase to either Sxl or π reduces its binding to the other site.

In the mother the da^+ locus produces an *excess* of da^+ factor which is stored in the egg. Following fertilization, the da^+ factor binds to ω; this results in the synthesis of a *small* quantity of repressor. Both male and female embryos have two copies fo ω and would therefore have the same quantity of repressor. In contrast, the number of Sxl and π sites is two each in the female and one each in the male. Repressor and RNA polymerase compete for binding to the low affinity Sxl and high affinity π sites. However, the affinity of the repressor for either of these sites is higher than that of the polymerase. Therefore the π site is preferentially bound by the repressor. Since the female has two π sites, these get bound to a significant extent by the repressor. However, repressor concentrations are limiting and therefore allow for polymerase binding to the low affinity Sxl sites; this leads to synthesis of significant amounts of the Sxl^+ product. In the male, on the other hand, there is only one copy each of the high and low affinity sites. The repressor thus practically saturates both these sites. Consequently, the low affinity Sxl site hardly binds RNA polymerase and little or no Sxl^+ product is produced. In a qualitative sense Sxl^+ product will therefore be made in the female but not in the male.

Our model also provides a ready explanation for the mutants discussed by Cline (1978). Eggs of da/da flies lack da^+ factor and therefore also lack repressor. In the absence of repressor, RNA polymerase binds preferentially to the π sites. As a result, affinity of the Sxl site for polymerase is reduced and thus a negligible amount of Sxl^+ product is produced. The Sxl^+ product being essential for females, this leads to the daughterless phenotype. The male lethal phenotype of Sxl^{M1} suggests that Sxl^+ product is produced in these males despite the low X/A ratio. Sxl^{M1} also acts as a suppressor of the daughterless phenotype. Both these effects of this remarkable mutation have a simple explanation in terms of our model: the mutation increases the affinity of the Sxl site for RNA polymerase, more RNA polymerase binds to it than in the wild type, and this leads to male lethality and survival of the daughters of da/da mothers.

A situation in which two sites compete for repressor and RNA polymerase resulting in the regulation of transcripion from these sites, is in fact known to exist in the bacteriophage lambda (Ptashne *et al.,* 1976, 1980; Walz *et al.,* 1976) where operator-promoter complexes for the genes *cro* and *cl* are close to each other and

are regulated by the same repressor. The *cro* operator-promoter complex, analogous to the π site in our model, has a higher affinity for repressor and RNA polymerase than the *cI* operator-promoter complex, analogous to the *Sxl* site. As a result, transcription from the *cI* promoter first increases and then decreases as a function of repressor concentration. In our model transcription from the *Sxl* site behaves in an identical fashion, also as a function of repressor concentration, and this leads to the model's many interesting features (see below).

Quantitative aspects

Relationship of level of Sxl+ product to viability: Since *Sxl+* product is assumed to regulate the rate of transcription of the X chromosomes, the level of *Sxl+* product per X chromosome is used here as the standard of comparison among different genotypes. We assume that the viability of a genotype varies with the level of *Sxl+* product per X chromosome and that a male is maximally viable at levels of *Sxl+* product lower than that at which females are maximally viable. Males are assumed to be inviable at levels of *Sxl+* product above those in the intersex and females at levels below. Clearly, a number of factors other than the level of *Sxl+* product must be contributing to the reduced viabilities of metamales (AAAXY), metafemales (AAXXX) and intersexes (AAAXX). However, we assume that the only effect in terms of the contribution of *Sxl+* product is that increasing levels of *Sxl+* product reduce male viability and decreasing levels reduce female viability. This assumption is consistent with the observation that *Sxl^{F1}* males (presumably with no *Sxl+* product) are fully viable as are *Sxl^{M1}/Sxl^{M1}* females (Cline, 1978). Thus we define levels of *Sxl+* product above those occurring in the intersex as the region of female viability. Conversely levels of *Sxl+* product below those in the intersex are defined as the region of male viability. It should be noted that this represents a modification of Cline's (1978) 'all-or-none' assumption that *Sxl+* product is essential for females and lethal for males.

Computation of levels of Sxl+ product: The calculations made in this paper refer to the binding equilibria between repressor and polymerase on the one hand and the low (*Sxl*) and high (π) affinity sites on the other. Binding is assumed to be Michaelian (non-cooperative) except that polymerase binding to either *Sxl* or π depresses its binding affinity to the other. Details are given in the legend to figure 2. The values of the parameters used as well as the range within which each can vary without affecting our conclusions are given in table 1. The result of these calculations is an expression for the equilibrium binding of RNA polymerase to the low affinity *Sxl* site. We assume that the level of this binding is directly reflected in the level of *Sxl+* product within the cell.
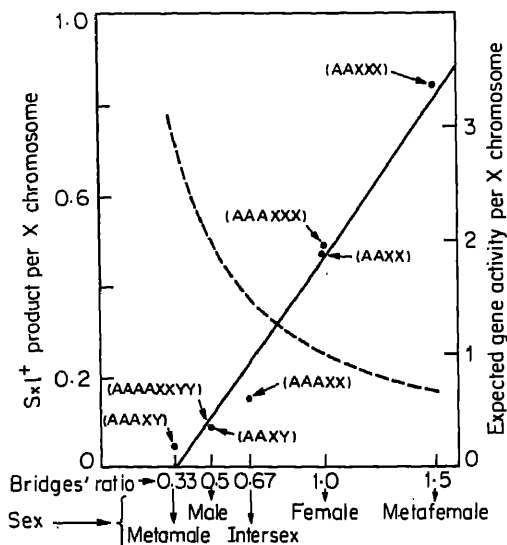
Figure 2. Levels of Sxl^+ product per X chromosome (left ordinate and solid line) and expected values of gene activity per X chromosome locus (right ordinate and broken line) (Chandra, 1979); both are expressed as a function of Bridges' ratio. Levels of Sxl^+ product were computed by solving for the following binding equilibria: $K_{LR} \times [L] \times [R] = [LR]$; $K_{LP} \times [L] \times [P] = [LP]$; $K_{HR} \times [H] \times [R] = [HR]$; $K_{HP} \times [H] \times [P] = [HP]$; where, [L], [H], [R] and [P] denote the free concentrations of low affinity sites (Sxl), high affinity sites (π), repressor, and RNA polymerase, respectively, and [LR], [LP], [HR] and [HP] are the concentrations of the bound complexes. The affinities of the reactions are denoted by K_{LR}, K_{LP}, K_{HR} and K_{HP} respectively.

The variable affinity in the binding of RNA polymerase to the two sites is simulated using the following equations:

$$K_{LP} = \frac{K_{LP}^o}{1 + \left(A \dfrac{[HP]}{[H_o]}\right)^n} \quad ; \quad K_{HP} = \frac{K_{HP}^o}{1 + \left(A \dfrac{[LP]}{[L_o]}\right)^n}$$

where, K_{LP}^o is the affinity of RNA polymerase to the Sxl site when no polymerase is bound to the π site, K_{HP}^o is the affinity of RNA polymerase to the π site when no polymerase is bound to the Sxl site; $[L_o]$, and $[H_o]$ are the total concentrations of Sxl and π; and A and n are constants. Thus, as binding of polymerase to the π site increases, K_{LP} decreases; when a fraction 1/A of the π sites is bound by polymerase, K_{LP} becomes half of K_{LP}^o and, finally, when all the π sites are occupied by polymerase, the affinity falls by a factor of $1 + A^n$. Binding of polymerase to Sxl reduces its affinity for π in a like manner.

Conservation conditions yield the following set of equations: $[LP] + [LR] + [L] = [L_o]$; $[HP] + [HR] + [H] = [H_o]$; $[LR] + [HR] + [R] = [R_o]$; $[LP] + [HP] + [P] = [P_o]$; where, $[R_o]$ and $[P_o]$ are the total concentrations of repressor and polymerase respectively.

For various sets of constants, $[L_o]$, $[H_o]$, $[R_o]$, $[P_o]$, K_{HR}, K_{LR}, K^o_{HP}, K^o_{LP}, A and n, the above equations were solved iteratively. $[L_o]$ and $[H_o]$ were taken as unity and scaled with the number of X chromosomes while $[R_o]$ and $[P_o]$ were scaled with the number of sets of autosomes. After these computations were completed, the gene coding for RNA polymerase II was shown to be on the X chromosome (Greenleaf *et al*.1980).$[P_o]$was therefore also scaled with the number of X chromosomes and the results do not alter any of our conclusions. The values of the various parameters used are given in table 1.

Table 1. Values of parameters used and their range of tolerance.

	Value used in the calculation	Range tolerated*
Repressor (R_0)	1.5	1.3 −1.7
RNA Polymerase (P_0)	5	2 −10
Affinity of polymerase for the low affinity site (K°_{LP})	1	0.5 −2.0
Affinity of polymerase for the low affinity site in the Sxl^{M1} mutation	10	5 −40
Affinity of polymerase for the high affinity site (K°_{HP})	100	10 −500
Affinity of repressor for the low affinity site (K_{LR})	100	50. −150
Affinity of repressor for the high affinity site (K_{HR})	10^8	2.5×10^4 −∞
A†	3.00	2.75 −3.50
n†	4.00	3.75 −4.50

* When the value of any parameter is outside this range, either the level of Sxl^+ product does not increase as a function of Bridges' ratio or the mutants do not behave as described in the text.

† These are constants in the equations used to simulate the variable affinity in the binding of RNA polymerase to the high and low affinity sites. See legend to figure 2 for details.

Numerical results

General Remarks

The level of Sxl^+ product per X chromosome increases in proportion to Bridges' ratio (figure 2). Triploids and tetraploids are extremely close to their diploid counterparts in their levels of Sxl^+ product per X chromosome, suggesting that it is indeed the X/A *ratio* rather than the level of X or A separately that is being measured. By applying the criteria for viability given earlier, one can see that (i) *da* is lethal in the female but not in the male; (ii) Sxl^{M1} is lethal in the male but not in the female; (iii) either one or two doses of Sxl^{M1} will rescue the daughters of *da/da* mothers and (iv) Sxl^{F1} is recessive because the level of Sxl^+ product per X chromosome in an Sxl^{F1}/Sxl^+ individual, though half of the normal level, is still within the region of female viability (table 2).

Mutations that can arise in the system

By varying the values of the parameters used beyond the assumed limits of viability of the wild type we are able to predict the kinds of mutations that can arise in this system (table 1). Many interesting consequences follow from the observation that the level of Sxl^+ product, when assessed as a function of increasing repressor concentration, first goes up and then comes down (figures 3A and B). Thus, whereas the region of female viability is confined to one continuous interval of repressor concentrations, males survive only at very low or high repressor levels.

Table 2. *Sxl+* product per X chromosome in wild type and mutant flies. ·

	Genotype	*Sxl+* product per X chromosome*
1.	*Wild type male*	0.08
2.	Sons of *da/da* mothers	0.10
3.	*Sxl^{M1}* male	0.40
4.	2, above, with *Sxl^{M1}*	0.52
5.	*Wild type female*	0.47
6.	Daughters of *da/da* mothers	0.09
7.	*Sxl^{M1}/Sxl^{M1}* female	0.68
8.	6, above, with *Sxl^{M1}/Sxl^{M1}*	0.47
9.	6, above, with *Sxl^{M1}/Sxl+*	0.28
10.	*Sxl^{F1}/Sxl+* female	0.24
11.	*Intersex* (AAAXX)	0.15
12.	Intersex offspring (AAAXX) of *da/da* mothers	0.13
13.	11, above, with *Sxl^{M1}/Sxl^{M1}*	0.48
14.	12, above, with *Sxl^{M1}/Sxl^{M1}*	0.60
15.	*Metamale* (AAAXY)	0.05
16.	15, above, with *Sxl^{M1}*	0.34
17.	*Metafemale* (AAXXX)	0.84
18.	Metafemale offspring (AAXXX) of *da/da* mothers	0.08

* These values were calculated as described in legend to figure 2. The parameters used are those listed in table 1.

Thus we have the apparently paradoxical situation in which a partial reduction in repressor levels leads to male-specific lethality, whereas totally eliminating the repressor restores viability to the male but results in female lethality. These predictions are mirrored respectively in the male-specific autosomal lethal, *mle* (Belote and Lucchesi, 1980a, b; Fukunaga *et al.*, 1975; Tanaka *et al.*, 1976) and in the *da* mutation (Cline, 1978). The other autosomal, male-specific lethals *msl-1*, *msl-1*[b], *msl-2* (Belote and Lucchesi, 1980a, b) are expected to be similar to *mle* in this respect.

The basis for the curious interaction between the *da* and *Sxl^{M1}* mutations is also brought out by Figs. 3A and B. *Sxl^{M1}* increases the level of *Sxl+* product above that of the wild type over the entire range of repressor concentrations. This has the effect of making the male nonviable over the entire range and of restoring viability to those females in which the repressor concentration is *simultaneously* lowered to near zero levels. *Sxl^{M1}*, therefore, is a male-specific lethal mutation which also has the property of rescuing the daughters of *da/da* mothers.

The observation that the level of *Sxl+* product first increases and then decreases with repressor concentration is central to explaining yet another curious result. This is the recent finding of Skripsky and Lucchesi (1980) that females of the genotype *mle/mle; Sxl^{F1}/Sxl+* develop, with a low penetrance, male secondary sexual characteristics (sex-combs). Referring to figure 3, the bell-shape of the curve implies that if the effect of the *mle* mutation is to partially reduce repressor concentration, it would lead to unacceptably high levels of *Sxl+* product in the male. In the female, on the other hand, the levels are slightly reduced but still within the region of viability. In combination with one dose of *Sxl^{F1}*, which by itself reduces the level of *Sxl+* product by one half, *mle/mle* would further lower the
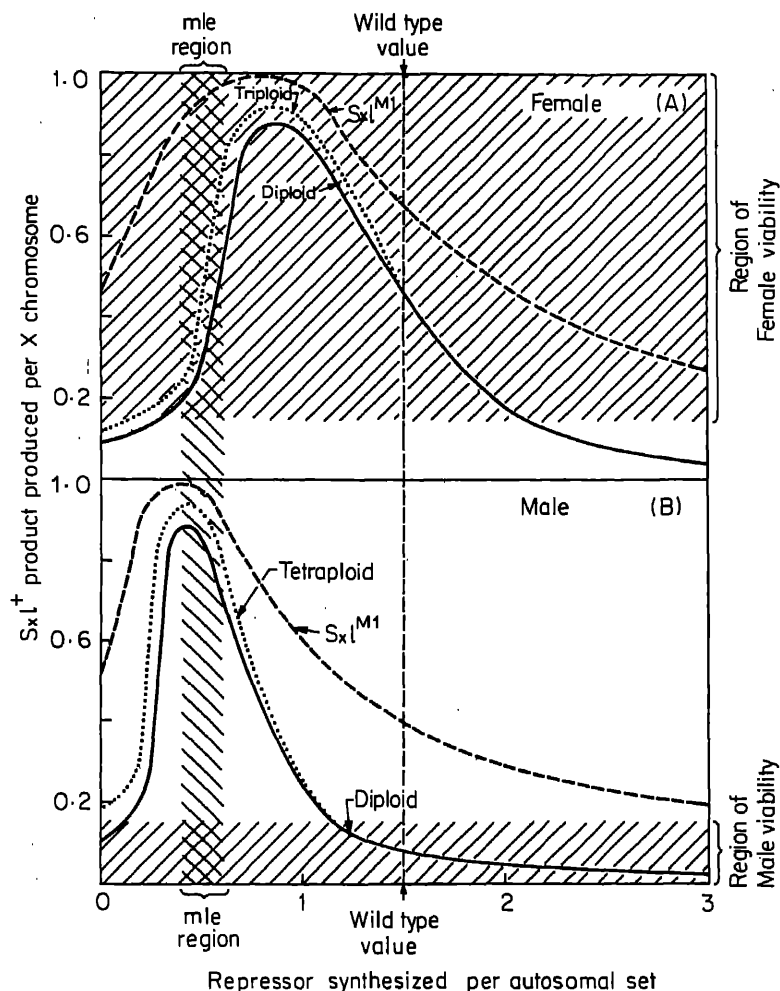
Figure 3. Levels of Sxl^+ product per X chromsome as a function of repressor synthesized per autosomal set in the female (A) and male (B).

Solid lines refer to the wild type, dotted lines to tetraploid male and triploid female and broken lines to the Sxl^{M1} male and female. The levels of Sxl^+ product corresponding to the regions of male and female viability and also the levels of repressor corresponding to the *mle* mutation are shown as hatched areas. Note that the range in levels of Sxl^+ product tolerated by a male is much narrower than that tolerated by a female. Metafemales have an extreme female phenotype and a rate of transcription per X chromosome lower than that in the normal female (Lucchesi *et al.* 1974; Stewart and Meriam, 1975). In terms of our model metafemales should have a level of Sxl^+ product higher than that of a normal female. Thus the level in normal females is expected to be somewhat below 1.0, the theoretical maximum value for Sxl^+ product per X chromosome. Sxl^{F1} is a recessive mutation (Cline, 1978). Since Sxl^{F1}/Sxl^+ females (presumably with half the wild type levels of Sxl^+ product) are fully viable, the level of Sxl^+ product in an intersex should be less than 0.5. The male, as a result, can only have a relatively narrow region of viability, ranging from zero to some value below 0.5 of Sxl^+ product per X chromosome. The *da* mutation corresponds to a zero level of repressor. The value of $[R_a] = 1.5$, considered as the wild type value, is indicated by the broken vertical line running through both panels.

level of Sxl^+ product and bring it to the neighbourhood of the male value. Consequently, such flies, if they survive, ought to show male-like characters.

The consequences of varying the affinities of the Sxl and π sites for repressor and RNA polymerase have been examined by us. A summary of these results and the properties of the various mutations, known as well as predicted, and the interactions among them are given in·summary form in table 3 and figure 4.

Table 3.   Properties of mutations, known and predicted.*

Mutation	Reference	Phenotype	Expected change at the molecular level	Can be rescued by	Will rescue the effects of
Sxl^{F1}	Cline (1978)	Female lethal	Inactive Sxl product	None	Sxl^{M1}, mle
da	Cline (1978)	Daughterless	No repressor, leading to low levels of Sxl^+ product in females	Sxl^{M1}	K_{LR} low
mle	Belote and Lucchesi (1980a, b); Fukunaga et al. (1975); Tanaka et al. (1976)	Male lethal	Lower than normal levels of repressor, leading to high levels of Sxl^+ product in males	Sxl^{F1}, K_{HP} high or K_{HR} low	None
Sxl^{M1} or K_{LP} high	Cline (1978); also see figure 4A	Male lethal	Higher than normal levels of Sxl^+ product in males	Sxl^{F1}	K_{HP} high, K_{HR} low, da
K_{LR} low	Predicted, see Fig. 4B	Male lethal	Higher than normal levels of Sxl^+ product in males	da	K_{HP} high, K_{HR} low
K_{HP} high	Predicted, see figure 4C	Female lethal	Lower than normal levels of Sxl^+ product in females	Sxl^{M1}, K_{LR} low	mle
K_{HR} low	Predicted, see figure 4D	Female lethal	Lower than normal levels of Sxl^+ product in females	Sxl^{M1}, K_{LR} low	mle
$\omega-$	Predicted	Female lethal	No repressor, leading to lower than normal levels of Sxl^+ product in females	Sxl^{M1}	K_{LR} low

* K_{LP} and K_{LR} are, as defined in legend to Figure 2, the affinities of the Sxl site to RNA polymerase and repressor respectively; similarly, K_{HP} and K_{HR} are the affinities of the π site to the polymerase and repressor respectively. The mutation K_{LP} *high* is one which results in an increase in K_{LP}; K_{LR} *low* is a mutation which results in a decrease in K_{LR}; K_{HP} *high* results in an increase in K_{HP} whereas K_{HR} *low* leads to a decrease in K_{HR}.

Changes in the affinity of the repressor or polymerase to these sites can be brought about by mutations in the sites themselves or by mutations affecting the properties of the repressor or polymerase. The former would behave as X-linked mutations and cannot be rescued by injection of cytoplasm from wild type eggs into defective eggs while the latter would behave as autosomal mutations and can be rescued by injection of cytoplasm from wild type eggs. The mutation Sxl^{M1}, which results in an increase in K_{LP}, is expected to be of the former kind because it is X-linked.
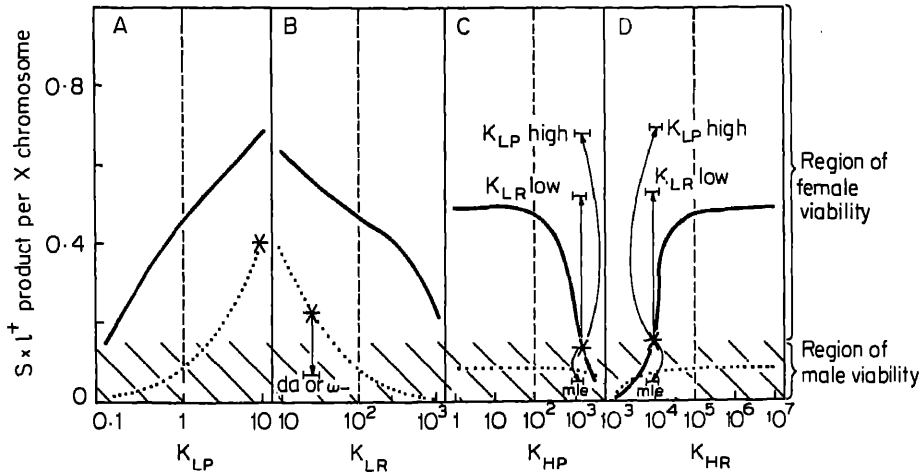
Figure 4. Levels of *Sxl⁺* product per X chromosome in the female (solid line) and male (dotted line) as a function of variations in the values of K_{LP} (Panel A), K_{LR} (Panel B), K_{HP} (Panel C) and K_{HR} (Panel D).

The panels A, B, C and D illustrate respectively the consequences of the four mutations *K_{LP} high*, *K_{LR} low*, *K_{HP} high* and *K_{HR} low*. The values considered as wild type for each of these affinities are indicated by broken vertical lines running through the middle of each panel. The values considered as mutant for each of these affinities are indicated by an asterisk in each panel. The region of male viability is indicated by the hatched areas whereas the region of female viability is unhatched. The two male-specific lethal mutations *K_{LP} high* and *K_{LR} low* can rescue females carrying either of the two female-specific lethal mutations *K_{HP} high* and *K_{HR} low* by restoring high levels of *Sxl⁺* product in them. This is indicated by means of long vertical arrows in panels C and D. On the other hand neither of the two female-specific lethals can rescue the male-specific lethals *K_{LP} high* and *K_{LR} low* because they do not bring about any significant reduction in the high levels of *Sxl⁺* product occurring in such genotypes. As shown by the arrow in Panel B, the mutations *da* and *ω* rescue males carrying the male-specific lethal mutation *K_{LR} low* by bringing down the amount of *Sxl⁺* product to a level at which males are viable. The *mle* mutation fails to rescue females carrying either of the two female-specific lethal mutations (*K_{HP} high* and *K_{HR} low*) because it further brings down the already low level of *Sxl⁺* product. This is shown by the short arrows in panels C and D. In all panels, the arrows indicate the levels of *Sxl⁺* product reached as a result of combining the mutation denoted against the arrow with the mutation illustrated in the panel. The values of affinities used here to represent the different mutations are arbitrary. See also table 3.

Discussion

The model discussed here provides a molecular mechanism for understanding how the X/A ratio can be measured in the cells of a developing embryo. The measurement is effected by means of a series of interactions initiated by the *da⁺* factor which result in a characteristic levels of *Sxl⁺* product in the cell. This *Sxl⁺* product is assumed to function as an inhibitor in regulating the rate of transcription of X-linked genes (Cline, 1978).

We wish to leave open the question whether the *Sxl⁺* product regulates transcription of the X chromosome *en bloc* or whether there are several *Sxl⁺*-like products regulating transcription in different sets of X-linked genes (see Chandra, 1979 for a review). We have also not discussed the consequences of duplications and deletions of the *Sxl* locus (Cline, 1978) because we do not know whether the

duplications and deletions include both the *Sxl* and π sites or not. Since the model requires that the two sites, *Sxl* and π, function in a coordinated fashion, it is not possible to predict the consequences of separating them. Nor have we discussed the results of Stewart and Merriam (1975) which seem to suggest that in flies with 2½ X chromosomes the relationship between dosage compensation and the Bridges' ratio breaks down irrespective of which chromosome arm is retained as the extra segment. These data cannot be simply interpreted in terms of our model because we do not know if the postulated relationship between *Sxl+* product and the Bridges' ratio (figure 2) also breaks down in these flies. It is possible that while this relationship is retained in such flies, breakdown occurs at the level of regulation of the rate of transcription by the *Sxl+* product. Resolution of this problem will depend to a significant extent on our understanding whether the X chromosome is regulated in a piecemeal or *en bloc* manner.

We now wish to make a few remarks regarding the implications of our model for the broader problems of dosage compensation and sex determination. We postulate that increasing levels of *Sxl+* product promote a female phenotype and, correspondingly, decreasing levels, a male phenotype. Independently of its effect on sexual phenotype, increasing levels of *Sxl+* product per X chromosome would lead to decreasing levels of X-linked gene products. Thus we consider the *Sxl+* product as having two primary roles, one in determining the sexual phenotype and the other, in dosage compensation. There are several other genes affecting sex determination (Baker and Ridge, 1980). The picture we have is that the *Sxl+* gene product initiates the pathway determining the sexual phenotype and that the other genes act subsequently.

Three predictions can be made about the role of the *Sxl+* product in dosage compensation. (i) Flies carrying the mutation *SxlF1* should have little or no *Sxl+* product. The rate of transcription of the X chromosome in such flies should therefore be higher than that in individuals carrying the wild type allele. This is consistent with the recent observations of Lucchesi and Skripsky (1981). (ii) Flies carrying the mutation *SxlM1* should have higher levels of *Sxl+* product than wild type individuals (table 2). The rate of transcription of the X chromosome in such flies should therefore be lower than in their wild type counterparts. Lucchesi and Skripsky (1981) have studied males of this genotype but their data did not permit them to distinguish between a lower rate of transcription and under-replication of the X chromosome. (iii) We expect the mutation *mle* to interfere with dosage compensation in the male by lowering the rate of transcription of the X chromosome; in the female, on the other hand, this mutation should have little or no effect (figures 3A and B). This is consistent with recent experimental data (Belote and Lucchesi, 1980a).

Three classes of data have a bearing on the relationship between *Sxl+* product and sex determination. One has to do with the sexual phenotype of flies carrying one or more of the mutations which form components of our model. For example, if *Sxl+* product is also involved in sex determination, we would predict that the sexual phenotype of (i) *SxlM1/SxlM1* and *SxlM1/Sxl+* females would shift in the direction of metafemales; (ii) *SxlF1/Sxl+* females would shift in the direction of

intersexes; and (iii) daughters of *da/da* mothers rescued by a single copy of *Sxl^M1* would more closely resemble intersexes than those rescued by two copies of *Sxl^M1*. A second class has to do with the effects of mutations which modulate the level of *Sxl^+* product in flies which already have an abnormal sexual phenotype. For example, an individual of the constitution AAAXX, which would normally develop as an intersex, might be expected to develop as a male under the influence of *da* and as a female under the influence *Sxl^M1* (table 2). This is in fact the observation of Cline (1981). Reasoning along the same lines, we predict that *da* would decrease the 'femaleness' of a metafemale while *Sxl^M1* would decrease the 'maleness' of a metamale along a male-female continuum (table 2). The third class of results pertains to the sexual phenotype in islands of mutant cells of one sex in a genetic background consisting of wild type cells of the other sex. In gynandromorphs, or in mosaics with viable *Sxl^M1* male tissue in a background of *Sxl^M1/Sxl^+* female tissue, our model suggests that the male tissue should exhibit phenotypic features of a female. Similarly, when viable, *Sxl^F1/Sxl^F1* female tissue within a *Sxl^F1/Sxl^+* background would show male characteristics. These are indeed the observations reported by Cline (1979a, b).

Finally, the following predictions can be made about the interaction of *Sxl^F1* with *Sxl^M1* and *mle*. Males carrying the mutations *Sxl^M1* or *mle* are inviable because, according to the model, there would be an overproduction of the *Sxl^+* product. *Sxl^F1* is assumed to be a mutation in the structural part of the *Sxl* locus leading to the production of inactive *Sxl* product. Since *Sxl^F1* males — which presumably have no *Sxl^+* product at all — are viable, it follows that *Sxl^F1* should rescue male embryos carrying *Sxl^M1* or *mle*. This prediction appears to be confirmed by the behaviour of two new alleles at the *Sxl^F1* region (Cline, 1981). Both these mutant alleles rescue *Sxl^M1* males from lethality. Data are not yet available for the interaction of *mle* with *Sxl^M1*.

We wish to point out that while some of the predictions made here are a direct consequence of the molecular mechanism we have proposed for the measurement of the X/A ratio, others follow from our quantitative approach to Cline's qualitative model for the behaviour of the *Sxl* and *da* mutations (Cline, 1978).

To account for certain experimental observations on levels of alcohol dehydrogenase activity in maize, Schwartz (1971) has proposed a 'gene competition' model which has certain features similar to our model for the measurement of the X/A ratio. According to Schwartz, the level of gene activity is related to the availability of a factor for which a group of genes competes. This factor, he assumes, is present in limiting concentrations. Schwartz's experimental results, which are consistent with this interpretation, suggest that such models are plausible in eukaryotic systems. Schwartz has in fact suggested that such a gene competition model may explain certain features of dosage compensation in *D. melanogaster* (Schwartz, 1973).

A feature of our model for dosage compensation is that it permits the measurement of *ratios* of the concentrations of two molecular species. This is brought out by the observation that the levels of *Sxl^+* product in the triploid female and the tetraploid male remain very close to those in their diploid counterparts throughout the range of repressor concentrations (figures 3A and B). In many

developing systems, the fate of a cell depends on its relative position with in the cell mass (Wolpert, 1971). A means for a cell to determine its relative position is to measure, for instance, the ratio of two substances ('morphogens') whose concentration gradients across the cell mass are in opposite directions. The consequences implied in looking at the problem of regulative development in such a manner are being examined by us.

Acknowledgements

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Abstract. The effect of early postnatal undernutrition and subsequent rehabilitation on wet weight, DNA, RNA, protein and the activities of acid and alkaline DNases in the cerebellar region of rat brain was studied. The cerebellar region was found to be affected significantly during early undernutrition. Further, earlier the initiation of nutritional rehabilitation the better was the recovery and in some cases timely nutritional rehabilitation resulted in better than normal biochemical composition of the brain. The specific activities of acid and alkaline DNases were not affected by early undernutrition. However, the total activities of these enzymes were significantly low in undernourished rats (R_{15} and R_{21}). Rehabilitation of these deprived groups upto 150 days resulted in higher amounts of these enzymes as compared to those of age-matched controls. It is concluded that the two DNases, are synthesized in a preferential manner during rehabilitation. It is further concluded that cerebellar region, in terms of development schedule and response to imposed calorie restriction, is intermediary between grey and white matter regions.

Keywords. DNA; DNases; undernutrition; rat cerebellum.

Introduction

Considerable evidence has accumulated in recent years to show that calorie/protein deprivation during early stages of development ('critical growth period') would lead to permanent biochemical deficiencies in the brain of various species of animals (Subba Rao, 1979; Subba Rao *et al.*, 1980) However, these studies were confined mostly to the changes in whole brain. It is becoming increasingly evident that different regions of brain have different schedules of development. Previous studies from this laboratory have shown developmental differences between grey and white matter regions (Subba Rao and Subba Rao, 1982a) and also the differential effects on these two regions of early undernutrition and subsequent rehabilitation (Subba Rao and Subba Rao, 1982b).

The cerebellar region of rat brain is known to develop during the early part of the postnatal period and hence represents another area of brain having a developmental time schedule different from both grey and white matter regions. We have therefore extended our earlier studies (Subba Rao and Subba Rao, 1982b) to examine certain biochemical parameters in rat cerebellum exposed to early postnatal undernutrition and subsequent rehabilitation. It is shown here that

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early undernutrition decreases the DNA, RNA and protein contents significantly in the cerebellar region whereas the specific activities of acid and alkaline DNases were unaffected. Rehabilitation of the undernourished animals upto 150 days corrected the deficiencies.

Materials and methods

Rats were fed 'rat feed' (Hindustan Lever, New Delhi) which is complete in all nutritional requirements. On the day of birth, the young rats were assigned to mothers in predetermined numbers. The control group (A) had 6 to 8 pups with the mother while the undernourished group (R) had 18 to 20 pups with the mother. Undernutrition was imposed on rats from the day of birth to varying periods, *viz.* 10, 15 and 21 days. After this restricted period, the animals were rehabilitated to normal conditions either by decreasing the litter size during the preweaning stage or by feeding *ad libitum* in the postweaning period. The rehabilitation was continued for varying periods. Such groups are designated as $R_{10}A_{15}$, $R_{15}A_{21}$, $R_{10}A_{50}$, etc., the first figure indicating the day upto which nutritional restriction was imposed and the second figure indicating the day upto which the rehabilitation was carried out, R and A representing restricted and adequate diets.

Animals were sacrificed by decapitation at various stages and the cerebellum was removed carefully. During the early stages *i.e.* 10, 15 and 21 days, 2 to 4 cerebella were pooled in order to obtain sufficient material. The tissue was homogenized with a Potter-Elvehjem type homogenizer in 9 volumes of cold distilled water. DNA and RNA were isolated according to the procedure of Schmidt and Thannhauser (1945) and were estimated by measuring the ultraviolet absorbance at 260 nm. The assay procedure for acid and alkaline DNases was described earlier (Shrivastaw and Subba Rao, 1975; Subba Rao and Subba Rao, 1982c). Protein was estimated by Lowry's method (Lowry *et al.*, 1951), while phosphorus was estimated by the procedure of Bartlett (1959).

Highly polymerized calf thymus DNA, yeast RNA and bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. All the reagents used were of Analytical grade. All the results are subjected to statistical treatment according to Student's 't' test.

Results and discussion

The effect of early undernutrition on the wet weight of rat cerebellum is presented in figure 1. As can be seen, nutritional deprivation reduced the wet weight significantly even at 10 days. Prolonged nutritional deprivation upto 21 days postnatal had no further effect on the wet weight. Rehabilitation of the under-nourished groups, R_{10} and R_{15}, upto 50 days restored the deficits to normal values, whereas if the rehabilitation was initiated at later date *i.e.* from 21 days postnatal (R_{21}) full recovery was not possible at 50 days. However, longer rehabilitation upto 150 days rectified the deficits in the wet weight.

In corollary with the changes in wet weights, the DNA content (figure 2a) is also significantly reduced by early postnatal undernutrition. At 21 days postnatal, the
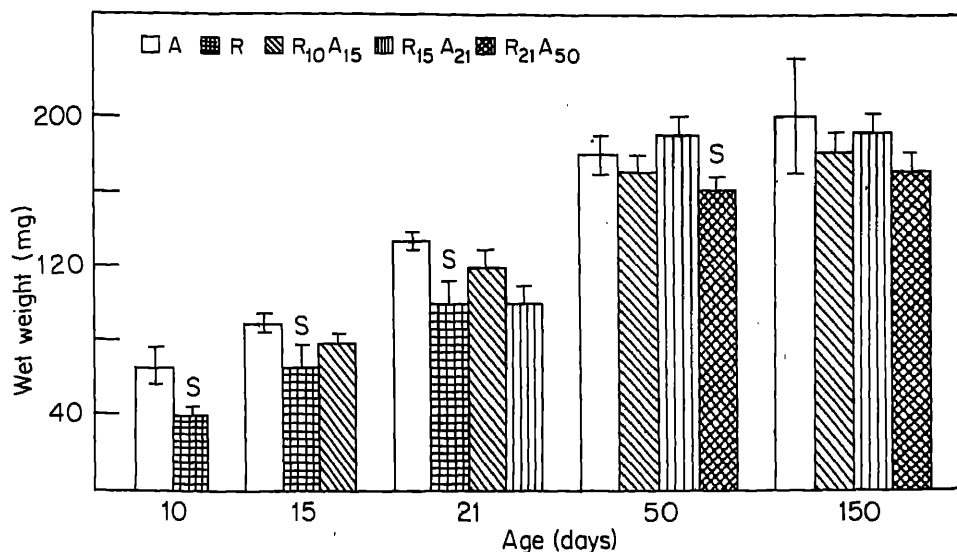
Figure 1. Effect of early postnatal undernutrition and subsequent rehabilitation on the wet weight of rat cerebellum.

A. Normal rats (viz. Normal 10 days old rat A_{10}; normal 15 days old rat A_{15} etc.). R. Nutritionally restricted rats. (viz. Undernourished from 1st day to 10th day postnatal R_{10}, undernourished from 1st day to 15th day postnatal R_{15}, etc.). $R_{10}A_{15}$. Rats undernourished from 1st day to 10th day postnatal and then rehabilitated for varying periods. (viz. Rats undernourished upto 10th day and then rehabilitated upto 15th day, $R_{10}A_{15}$; Rats undernourished upto 10th day and then rehabilitated upto 21 day $R_{10}A_{21}$, etc.). $R_{15}A_{21}$. Rats undernourished from 1st day to 15th day postnatal and then rehabilitated for varying periods. (viz. Rats undernourished upto 15th day and then rehabilitated upto 21st day $R_{15}A_{21}$, etc.) $R_{21}A_{50}$. Rats undernourished from 1st day to 21st day postnatal and then rehabilitated for varying periods. (viz. Rats undernourished upto 21st day and then rehabilitated upto 50th day $R_{21}A_{50}$, etc.).

All the values are expressed in mg. ±S.D.

The number of animals in each group varied from 6 to 8. For other details please see materials and methods section of text.

'S' These values are sigificantly different from the corresponding age matched controls (P<0.005).

undernourished group has only 50% of DNA as that of age matched control. However, the concentration of DNA expressed per g of tissue (figure 2b), was not affected by undernutrition at 10 and 15 days, whereas significant reduction in the concentration of DNA could be observed at 21 days. These results are in agreement with the earlier studies (Culley and Lineberger, 1968; Winick, 1970; Balazs and Patel, 1973; Gopinath *et al.*, 1976). As could be expected, the earlier the initiation of rehabilitation, the greater is the recovery seen. Thus when the R_{10} group was rehabilitated upto 21 days postnatal, the DNA value reached almost to normal values whereas R_{15} and R_{21} groups recovered to complete normalcy only after rehabilitating upto 50 days and 150 days respectively (figure 2a). It can also be noted that when the R_{10} group was rehabilitated either upto 50 days or 150 days

both concentrations as well as the total DNA contents showed remarkable recovery to values that were significantly higher as compared to the age matched controls.
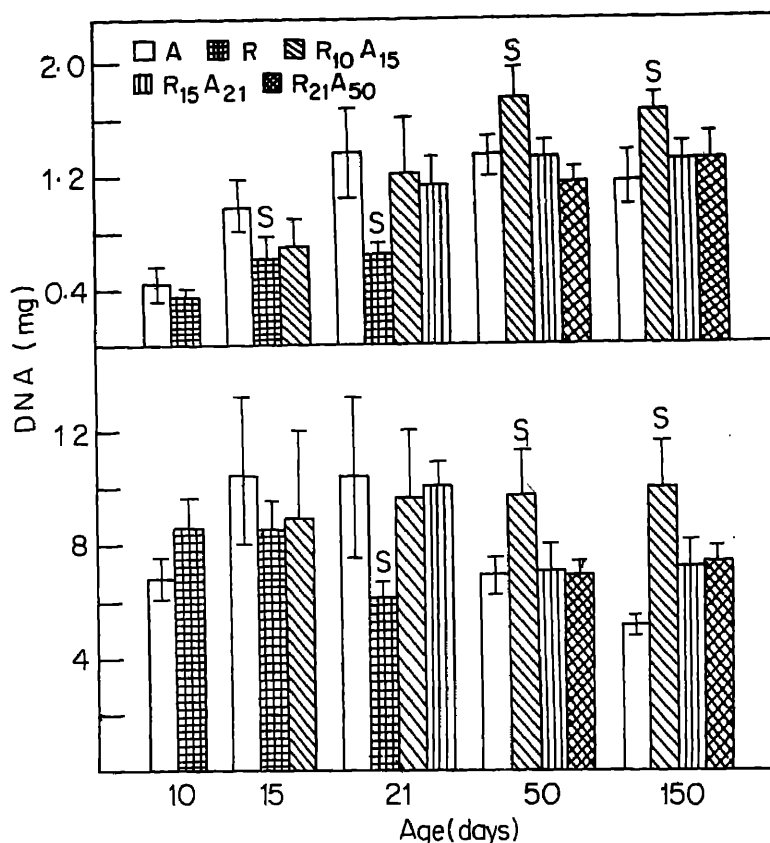
Figure 2. Effect of early postnatal undernutrition and subsequent rehabilitation on the DNA content of rat cerebellum.

(a) DNA content whole region.

(b) RNA content, expressed per gram of region.

A. Normal rats. R. Nutritionally restricted rats. $R_{10}A_{15}$, Rats rehabilitated from 10th day postnatal. $R_{15}A_{21}$, Rats rehabilitated from 15th day postnatal. $R_{21}A_{50}$, Rats rehabilitated from 21st day postnatal.

The number of animals in each group were the same as in figure 1. For other details please see under figure 1.

'S' These values are sigificantly different from the corresponding age matched controls (P<0.005).

The effect of early postnatal undernutrition followed by rehabilitation on RNA and protein contents of rat cerebellum are presented in figure 3 and 4. It is again clear that RNA and protein contents in undernourished rats reduced significantly (figures 3a and 4a). However, the concentration of RNA was not affected by undernutrition whereas the protein concentration was significantly reduced in 21 days undernourished rats (about 30%) (figures 3b and 4b). Rehabilitation of these
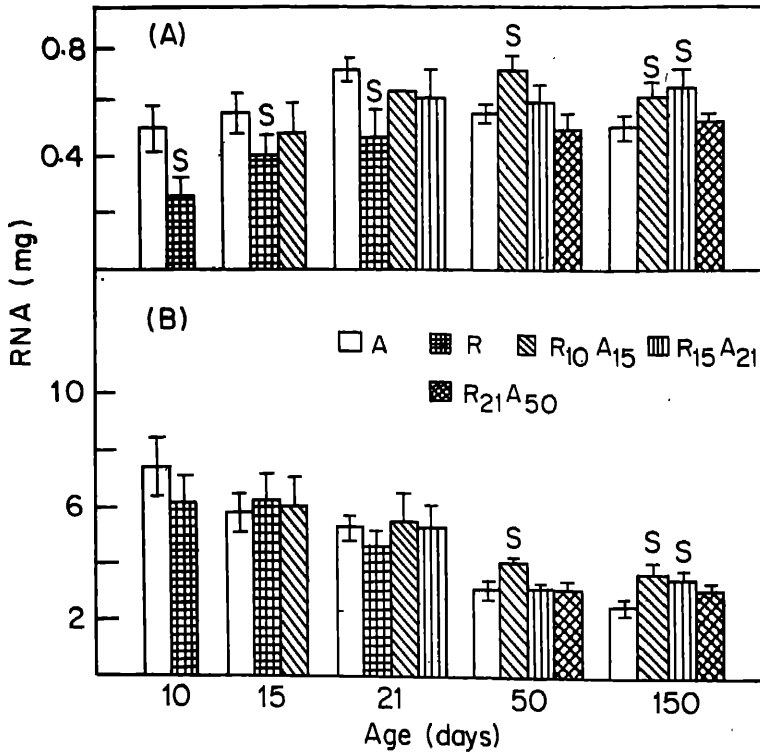
Figure 3. Effect of early postnatal undernutrition and subsequent rehabilitation on the RNA conent of rat cerebellum.

(A) RNA content per whole region.

(B) RNA content expressed per gram of region.

A. Normal rats. R, Nutritionally restricted rats. $R_{10}A_{15}$, Rats rehabilitated from 10th day postnatal. $R_{15}A_{21}$, Rats rehabilitated from 15th day-postnatal. $R_{21}A_{50}$, Rats rehabilitated from 21st day postnatal.

The number of animals in each group were the same as in figure 1. For other details please see under figure 1.

'S' These values are sigificantly different from the corresponding age matched controls (P<0.005).

undernourished groups R_{10}, R_{15} and R_{21} for different periods yielded varying extents of recovery of RNA and protein depending on the initiation and duration of nutritional deprivation and of rehabilitation. It can also be noted that the protein contents were significantly low in $R_{21}A_{50}$ group; however, rehabilitation upto 150 days ($R_{21}A_{150}$) resulted in full recovery (figure 3a). The RNA contents in rehabilitated groups ($R_{10}A_{150}$, $R_{15}A_{150}$) are significantly higher as compared to the age-matched controls (figure 3a). The above results show that as far as the DNA and RNA synthesis is concerned complete recovery is operative and such a compensatory mechanism does not seem to operate, however, in the case of protein.
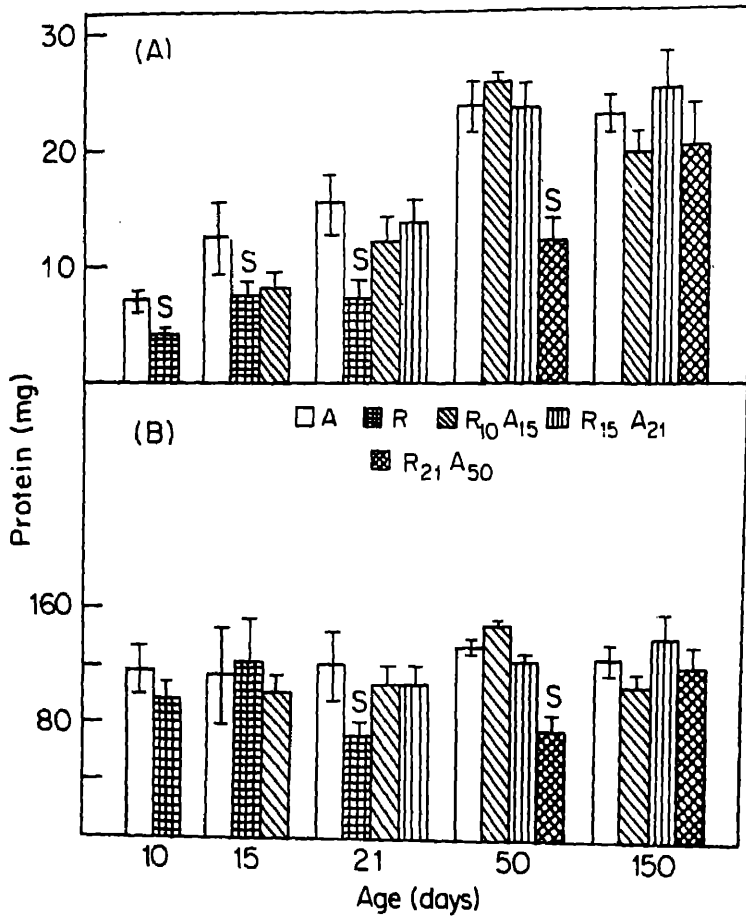
Figure 4. Effect of early postnatal undernutrition and subsequent rehabilitation on the Protein conent of rat cerebellum.

(A) Protein content per whole region.

(B) Protein content, expressed per gram of region.

A. Normal rats. R, Nutritionally restricted rats. $R_{10}A_{15}$, Rats rehabilitated from 10th day postnatal. $R_{15}A_{21}$, Rats rehabilitated from 15th day postnatal. $R_{21}A_{50}$, Rats rehabilitated from 21st day postnatal.

The number of animals in each group were the same as in figure 1. For other details please see under figure 1.

'S' These values are sigificantly different from the corresponding age matched controls (P<0.005).

Since earlier studies from this laboratory revealed a positive correlation between DNA content and the activities of two putative DNA degrading enzymes, acid and alkaline DNases in chick and rat brain (Shrivastaw and Subba Rao, 1975; Subba Rao and Subba Rao, 1982a), we also measured the activities of these enzymes in
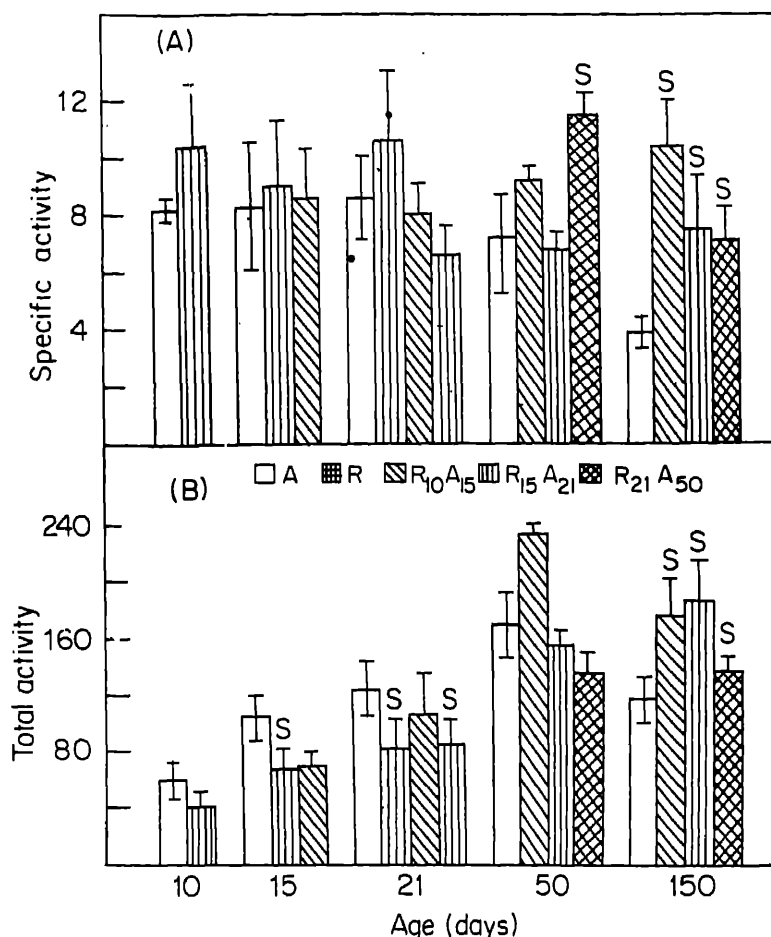
Figure 5. Effect of early postnatal undernutrition and subsequent rehabilitation on the activity of Acid DNase.

(A) Specific activity (μg-of DNA-P liberated per 2hr per mg of protein).

(B) Total activity, (Specific activity $\times$ Total Protein in mg)

A. Normal rats. B, Nutritionally restricted rats. $R_{10}A_{15}$, Rats rehabilitated from 10th day postnatal. $R_{15}A_{21}$, Rats rehabilitated from 15th day postnatal. $R_{21}A_{50}$, Rats rehabilitated from 21st day postnatal.

The number of animals in each group were the same as in figure 1. For other details please see under figure 1.

'S' These values are significantly different from the corresponding age matched controls (P<0.005).

the present group of rats. The results obtained are presented in figures 5 and 6. The specific activity of acid DNase (figure 5a) did not change as a result of postnatal undernutrition. The total activities, however, showed significant decrease in

undernourished rat cerebellum at 15 and 21 days of postnatal age (figure 5b). In all these cases rehabilitation beginning from 10th, 15th or 21st day upto 150 days resulted in activities (both specific as well as total), which were markedly higher than those noticed in corresponding age-matched controls. The results concerning the alkaline DNase activity (figure 6) are similar to that of acid DNase. However,
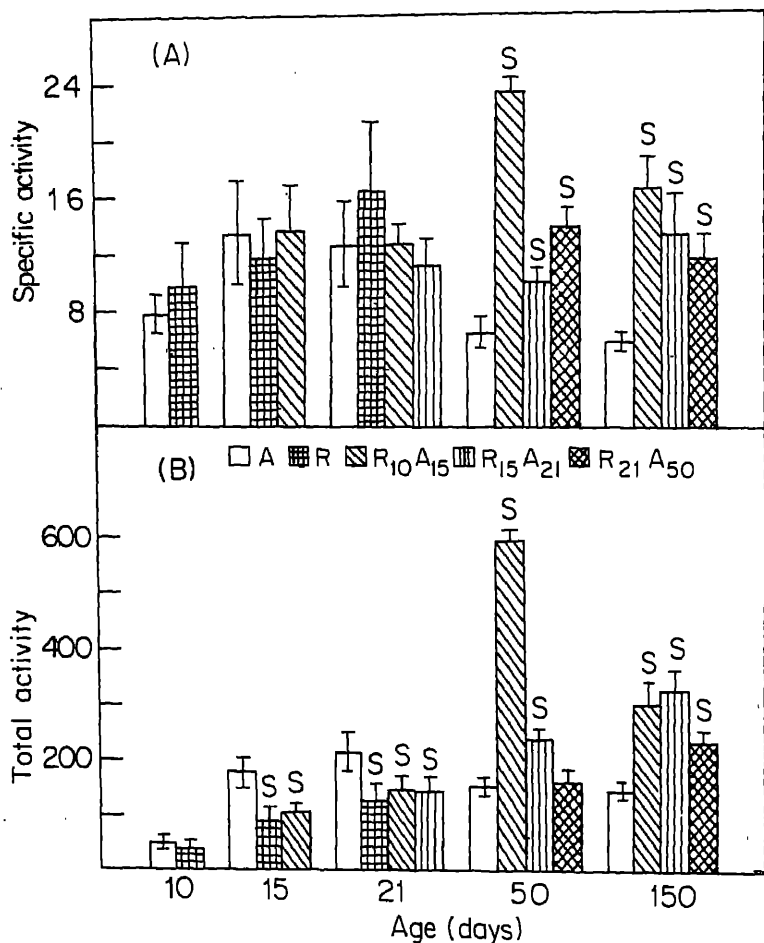
Figure 6. Effect of early postnatal undernutrition and subsequent rehabilitation on the activity of alkaline DNase.

(A) Specific activity (μg of DNA-P liberated per 2hr per mg of protein).

(B) Total activity (specific activity $\times$ total protein in mg).

A. Normal rats. R, Nutritionally restricted rats. $R_{10}A_{15}$, Rats rehabilitated from 10th day postnatal. $R_{15}A_{21}$, Rats rehabilitated from 15th day postnatal. $R_{21}A_{50}$, Rats rehabilitated from 21st day postnatal.

The number of animals in each group were the same as in figure 1. For other details please see under figure 1.

'S' These values are significantly different from the corresponding age matched controls ($P<0.005$).

in the case of alkaline DNase the rehabilitation of groups R_{10} to 50 days ($R_{10}A_{50}$, $R_{15}A_{50}$) could itself bring significantly higher activities (both specific and total) as compared to the corresponding age-matched controls (figures 6a, 6b). These results confirm the earlier observation from this laboratory on white and grey matter regions of rat brain (Subba Rao and Subba Rao, 1982b). It is of considerable importance to note that both the acid and alkaline DNase activities were unchanged in the undernourished rat cerebellum. In particular, the alkaline DNase activity seems to be conserved against the limited energy and protein available to the brain under experimental conditions. As can be seen from figure 6 levels of this enzyme are markedly high in rehabilitated animals ($R_{10}A_{50}$, $R_{15}A_{150}$) and this clearly suggests preferential synthesis of this enzyme during rehabilitation. Although DNases are supposed to be primarily degradative in nature in function, it is suspected now that these enzymes might be playing some important role either in the synthesis or repair of DNA. Thus earlier studies by Allfrey and Mirsky (1962), Gautier and Leonard (1962) have shown high levels of cellular DNases in a wide variety of organisms during the interval in the growth cycle when DNA synthesis is proceeding at maximal rate. Studies with purified DNA polymerase (Kornberg, 1964) revealed that nucleases can profoundly affect the template by providing required nicks, hence the rate of cell replication. It has also been shown by Yagi and Okamura (1965) that DNases serve for the excision of lesions introduced into the DNA as a result of exposure to UV irradiation or alkylating reagents thus permitting the repair of the impaired nucleic acid. On the basis of these experiments it has been proposed by Lehman (1967) that DNases might be playing a crucial role either in synthesis or repair of DNA. Later experiments by Bernardi (1971) and Slor *et al.* (1973) support the above contention. Our present results also support such a concept.

We have shown earlier that grey matter is unaffected and white matter is most affected by early undernutrition. The present studies further point out the intermediary nature of the cerebellar region in the developmental schedule and its response to nutritional deprivation and subsequent rehabilitation. Thus the cerebellar region is affected by calorie restriction but proper rehabilitation could correct the defects.

Acknowledgements

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Lipofuscin like compound in mango

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Abstract. Thin layer chromatographic separation of chloroform-methanol extracts of mango on silica gel revealed a fluorescent substance in mango peel and pulp. The compound had fluorescence spectrum similar to that of lipofuscin, the age pigment of animal tissues and was found to be water insoluble and stable to ultraviolet irradiation. The fluorescent material appeared to be a lipoprotein.

Keywords. Mango; lipofuscin; spectral characterization.

Introduction

Lipofuscin (age pigment) is known to accumulate in animal tissues during ageing and is extensively documented in literature on mammals (Reichel, 1971; Reddy *et al.*, 1973; Tauna, 1975). It is a compound formed by the complexation of protein with malonaldehyde derived by the peroxidation of poly-unsaturated lipids of subcellular membranes and is implicated in cellular damage due to lipid peroxidation and ageing (Hendley *et al.*, 1963; Porta and Hartraft, 1969; Herman, 1972). A lipofuscin like compound with typical fluorescent properties was reported in ripening pear and banana (Fletcher, 1973; Maguire and Haard, 1976). Recently a compound with fluorescent properties similar to lipofuscin was reported to be present in apples (Knee, 1982). It was of interest to see if such a compound accumulates in other fruits and we report the presence of a similar compound in mango peel and pulp.

Materials and methods

Fully ripe badami variety of mangoes were selected for this study. Freeze dried mango peel and pulp (5 g) respectively were extracted for 10 min at 50°C in 30 ml of chloroform-methanol mixture (2 : 1 v/v). The extract was filtered and evaporated to dryness under suction. The residue was dissolved in 3 ml of the chloroform-methanol mixture. An aliquot of this (0.3 ml) was spotted on a silica gel thin layer chromatography plate and developed with petroleum ether for 10 min. The plates were removed and were developed again with a mixture of petroleum ether − chloroform − methanol (3 : 1 : 1 − v/v) for a further period of 10 min. TLC

Abbreviations used: TLC, thin layer chromatography; UV, ultraviolet.

plates were exposed to ultraviolet light. The area on TLC plate at the origin containing the fluorescent compound was scrapped off the plate and the compound was extracted with chloroform-methanol (2 : 1 v/v). The extract was completely colourless and showed bright fluorescence when exposed to UV light. This was used for further studies. The fluorescence spectrum was recorded in a Perkin Elmer spectrophotofluorimeter. Quinine sulphate 0.1 μg in 0.1 N H_2SO_4, was used as a reference standard. The standard had a relative fluorescence intensity of 2.7. Both the excitation and emission spectra were obtained for the samples.

Lipid peroxidation was studied by the thiobarbituric acid method (Sidwell *et al.*, 1955; Holland, 1971). Protein (Lowry *et al.*, 1951), polyphenol (Swain *et al.*, 1959) and carotenoid estimations (Association of Vitamin Chemists, 1966) were carried out by the procedures indicated. Amino acids were separated by ascending paper chromatography on Whatman No. 2 paper using the following solvents (1) phenol-water-NH_3, 80 : 20 : 0.5 (v/v) (2) *n*-butanol-pyridine-water, 1 : 1 : 1 (v/v) and (3) *n*-butanol-acetic acid-water, 4 : 1 : 1 (v/v). The chromatograms were dried in air and the amino acids located by spraying with 0.5% ninhydrin in acetone and heating for 10 min at 60°C.

Results and discussion

In the procedure employed by Maguire and Haard (1976), the fluorescent measurements for the compound extracted from banana were carried out directly on the original chloroform-methanol mixture. The extracts from banana showed an excitation maximum at 350 nm and an emission peak around 440 nm (Maguire and Haard, 1976) which is the expected spectrum for lipofuscin. In the present study, however, the original extract of mango showed the excitation maximum at 350 nm but there was no emission in the range of 400–480 nm.
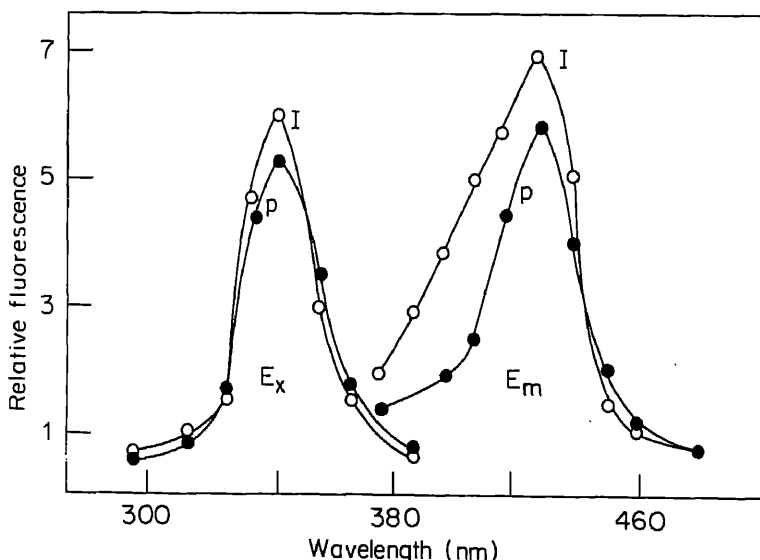
Figure 1. Excitation and emission spectra of fluorescent lipofuscin – like compound obtained after TLC separation.

To rule out the possibility of interference and quenching of fluorescence by the pigments present in the extract, as the original extract was highly coloured (0.3 ml of which contained 84 µg of total carotenoids in peel and 100 µg in pulp), it was necessary to remove the pigment from the extracts. After preliminary trials it was found that TLC on silica gel afforded a very good separation of the fluorescent compound from the pigments. Chromatography using petroleum ether followed by chloroform-methanol-petroleum ether was necessary for a clear separation. The fluorescent compound thus isolated showed excitation and emission maxima similar to that of lipofuscin *i.e.* 350 and 430 nm respectively (figure 1). Previous work has indicated the possibility of flavins and retinol-like compounds interfering with the estimation of the age pigment (Fletcher *et al.*, 1973). This interference is generally removed by washing the extract with water to remove phenolics and flavin like compounds, if any, and exposure of the extract to UV light to remove retinol and similar compounds (Fletcher *et al.*, 1973). Washing with water and UV exposure had no effect on the fluorescence spectrum of the compound isolated in the present study. Thus, it appears that in the case of coloured plant materials, removal of the colouring matter may be necessary to demonstrate the presence of the age pigment.

Recently (Knee, 1982) indicated that the procedure devised for lipofuscin estimation in animal tissues may not be applicable to plants because of the interference from phenolics and carotenoids. In the present study, by using TLC and using 2 different solvent systems, it has been possible to separate the lipofuscin-like compound from the interfering substances. The material thus separated was not a carotenoid or a phenolic compound but was a peptide. The fact that it is a lipid peroxidation product was confirmed by its positive reaction with thiobarbituric acid reagent. The hydrolysis of the fluorescent pigment fraction (Dowex-50 treated) yielded 7 amino acids separable by paper chromatography. However it is necessary to purify this lipoprotein to homogeneity for any further characterization and quantitization.

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Effect of repeated oral administration of quinalphos to male goat (*Capra hircus*)

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Abstract. Quinalphos given in daily oral doses of 0.5 mg/kg for 110 days induced severe signs of organophosphorus poisoning in male goats. The inhibition of acetylcholinesterase activity in erythrocyte was highly significant. The activity of liver glutamic; oxaloacetic transaminase, glutamic; pyruvic·transaminase, alkaline phosphatase and protein indicated marked alteration. The haematological changes were however, relatively less significant with the exception of a very low count of red blood cells and white blood cells in the treated animals. Among the vital organs, only liver suggested mild cellular changes due to quinalphos intoxication. There was no significant pathological change in other organs of the treated animals.

In animals observed after 15 and 30 days rest, the activity of acetylcholinesterase in red blood cells and haematological picture showed a fairly good recovery. This study suggests that although quinalphos in low concentrations did not produce discernible cellular changes, it induced highly significant enzymatic and haematological changes in the goat.

Keywords. Organophosphorous; acetylcholinesterase; erythrocyte; quinalphos.

Introduction

Hazards and environmental contamination through abuse of a variety of pesticides has attracted global attention. In recent years emphasis has been given to organophosphorous pesticides instead of organochlorine compounds to overcome the problem of residue. Quinalphos (O, O-diethyl-O-quinoxalin-2-yl phosphorothioate) due to its acaricidal and insecticidal properties is in large scale use in this country. From an annual consumption of 300 metric tons during 1977, the use has risen to 1000 metric tons (Anon 1978, 1979). Quinalphos is presently used in different formulations such as emulsion concentrates, granules and dust.

Single and also multiple feeding of quinalphos produced highly significant toxicological effects in rats (Dikshith T. S. S., Datta, K. K. and Raizada, R. B. unpublished). It also induced significant inhibition· in the activity of acetylcholinesterase in red blood cells (RBC) and brain of guinea-pigs (Dikshith *et al.,* 1980a). Rats pretreated with carbon tetrachloride were more susceptible to quinalphos intoxication (Dikshith *et al.,* 1980b). Response and toxic effects of

Abbreviations used: RBC, red blood cells; WBC, white blood cells.

pesticides have been associated with species differences. The mechanism of disposing of lipid-soluble toxic chemicals such as pesticides by different species of animals has already been reported (Murthy and DuBios, 1957; Hayes, 1959; Brodie and Maickel, 1962; Durham, 1967; Fouts, 1970 and Cantilema *et al.*, 1979). Since domestic goat is associated with the food chain of man and also represents the livestock animals the present study could help in identifying cases of organophosphorous poisoning among livestock animals and also in occupational workers handling these potentially toxic insecticides.

Materials and methods

Animals

Four domestic male goats (average body weight 16 kg) were procured from the breeders. The animals were under observation for one month before the commencement of medication with quinalphos. They were maintained on green leaves, grass and peeled husks of grains and were allowed to move freely in the field during the day unless otherwise mentioned.

Preparation of the compound and treatment

Technical quinalphos obtained from Regional Research Laboratory, Jorhat, was used in this study. The compound dissolved in peanut oil was orally administered daily to two male goats (0.5 mg/kg) for a period of 110 days. Two goats of the control group were administered equal volume of peanut oil in a similar manner. Blood was sampled periodically at 60, 90 and 110 days during treatment and 15 and 30 days during post-treatment rest periods in oxalated tubes (2 mg of 6 parts amonium oxalate and 4 parts potassium oxalate/ml blood) for acetylcholinesterase and haematoiogical assays. Experimental and control groups of animals were killed after 140 days of treatment.

Period of exposure and sampling

Earlier we have observed that low dose of quinalphos given to guinea-pigs for 30 days could not elicit significant toxicological effects (Dikshith *et al.*, 1980a). In the present study we therefore exposed the animals for much longer periods and also sampled after 60, 90 and 110 days after treatment. Since the treated goats became very weak after 110 days of exposure, further administration of quinalphos was stopped. The animals were under close observation till the experiment was terminated. Blood was also sampled after 15 and 30 days of post-treatment rest for the estimation of acetylcholinesterase activity.

Histological studies

Sections of the liver, kidney, adrenal, brain and testis were fixed in normal-saline solution (100 ml formalin + 900 ml distilled water + 8.5 g sodium chloride). After routine processing, paraffin sections were cut at 6 μm thickness and stained with haematoxylin-eosin (McManus and Mowry, 1960).

Biochemical studies

The liver was washed free of extraneous material using chilled saline solution. The

liver was homogenized in 0.25 M ice-cold sucrose solution (10% w/v) in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 700 g for 10 min and the supernatant was collected. Serum was separated. The supernatant and serum were used for estimation of enzyme activities. The activity of glutamic-oxaloacetic transaminase (E.C.2.6.1.1) glutamic-pyruvic acid transaminase (E.C.2.6.1.2) and alkaline phosphatase (E.C.3.1.3.1, orthophosphoric monoester phosphohydrolase) were determined by the method of Wootton (1964). Serum bilirubin was estimated by the procedure of Mallary and Evelyh, modified by Ducci and Watson (1945). The method of Lowry *et al.* (1951) was followed for the determination of protein while the Nelson-Somogyi (1965) method was adopted for blood sugar.

Acetylcholinesterase determination

Brain was quickly removed, washed free from extraneous material and homogenized in 0.25 M ice-cold sucrose solution 10% w/v in a Potter-Elvehjem type homogenizer. RBC was separated by centrifuging the blood at 2500 g for 10 min. Acetylcholinesterase (E.C.3.1.1.7) activity was assayed by the method of Hestrin (1949).

Haematological studies

Total RBC and total white blood cell (WBC) were determined by the method of Wintrobe and Landsberg (1935) and haemoglobin was estimated according to the method of Kolmer *et al.* (1951).

Results
Clinical signs

Male goats which received a daily dose of quinalphos (0.5 mg/kg) for a period of 110 days indicated body tremor, profuse salivation, weakness, diarrhoea and reduced food consumption. The animals became weak after 30 days of continuous exposure to quinalphos but there was no paralysis or death.

Microscopic examination

Liver showed fatty degenerative changes in the parenchyma. Hepatocytes of the centrolobular area appeared vacuolated and carried granular cytoplasm and the nuclei were pushed to a corner of the cell. However there was no necrosis of the liver (figure 1, 2). Other organs did not suggest any kind of morphological change due to quinalphos.

Biochemical studies

The activity of liver and serum glutamic-oxaloacetic, glutamic pyruvic transminases, alkaline phosphatase activities, protein content and serum bilirubin is shown in table 1. The activity of the transaminases, protein content as well as serum protein in the quinalphos treated animals showed a highly significant reduction ($p < 0.001$ to $p < 0.01$) in comparison to the value in the control animals. However the activity of alkaline phosphatase both in liver and serum was increased ($p < 0.01$). The level of serum bilirubin was very high ($p < 0.001$) in exposed animals (table 1), while that of blood sugar was comparable with the control.

Figure 1. Section of liver of control goat (H and E×164).
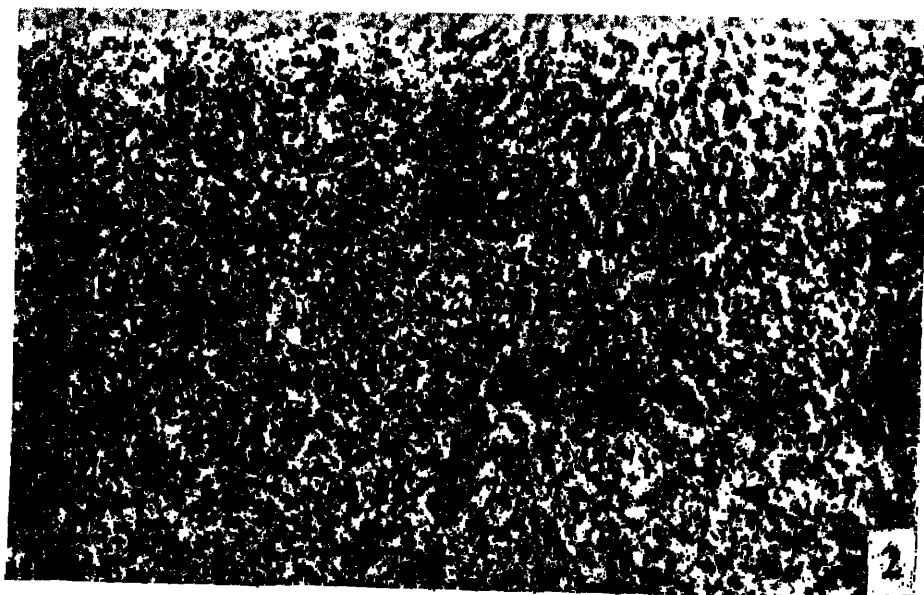
Figure 2. Section of liver of goat exposed to quinalphos (H and E×164).

Table 1. Biochemical changes in liver, serum and blood of male goats exposed to quinalphos.

Treatment	Control (peanut oil 0.5 ml/day)	Quinalphos (0.5 mg/kg/day)
Liver		
GOT	7.52±0.32	4.29^a±0.10
GPT	6.05±0.19	3.46^b±0.35
Alkaline phosphatase	0.26±0.17	1.912^b±0.19
Total protein	31.12±0.13	14.25^a±0.13
Serum		
GOT	0.04±0.003	0.30±0.003
GPT	0.013±0.002	0.019±0.001
Alkaline phosphatase	0.076±0.001	0.27^b±0.003
Total protein	23.42±0.23	14.6^a±0.17
Bilirubin	0.34±0.02	0.70^a±0.01

GOT — glutamic-oxaloacetic transaminase; GPT — glutamic-pyruvate transaminase; alkaline phosphatase (μmol/g or ml/min)

Total protein (mg/g or ml); Bilirubin — (mg/100 ml serum)

$a — p<0.001;$ $b — p<0.01$

Table 2. Activity of RBC acetylcholinesterase in male goats exposed to quinalphos.

Treatment (days)	Acetylcholinesterase (μmol/ml/10 min)
Control peanut oil (O)	5.85 ±0.12
Treatment	
60	2.84^a ±0.12
90	1.89^a ±0.09
110	0.82^a ±0.14
Post-treatment rest	
15	1.96^{b*}±0.06
30	3.38^{a*}±0.20

RBC — Red blood cells

a $p<0.001;$ $b$$p<0.01$

* Values significantly different in comparison to the value of 110 days exposure.

Acetylcholinesterase activity

Male goats exposed to quinalphos for 110 days showed significant reduction in acetylcholinesterase activity ($p<0.001$) in comparison with the control (0.82 compared to 5.85). Acetylcholinesterase after 15 days and 30 days of post-treatment rest period was 1.96 and 3.38 respectively indicating that a highly significant recovery in the acetylcholinesterase activity had occurred on stopping the treatment (compared to 0.82 of 110 days treatment) (table 2).

Haematological studies

Although goats treated with quinalphos for 110 days exhibited significant decrease ($p<0.001$) of RBC and WBC counts the values were within the normal range. There was a significant recovery of RBC and WBC counts after 15 and 30 days of post-treatment rest (table 3).

Table 3.   Haematological changes in male goats exposed to quinalphos.

Treatment (days)

	Control (peanut oil)				Post-treatment rest	
	0	60	90	110	15	30
RBC	15.21±0.15	13.42±0.96	14.45±0.27	10.9^a±0.21	14.92±0.19	20.52*±0.27
WBC	6800±170	7150±80	6000±260	3850^a±110	3950^a±192	6350±250
Hb	9.2±0.31	14.5±1.99	10.4±0.31	10.0±0.31	11.0±0.42	12.20*±0.31
N	39±0.22	4^a±0.57	55^a±0.57	40±0.57	35^a±0.67	32^a±0.57
L	58±1	44^a±0.51	43^a±0.57	59±0.57	58±0.67	67^a±0.57
M	3±0.57	2±0.5	2±0.5	.1±0.29	4±0.5	2.9

^{a} $p<0.001$; ^{b} $p<0.05$

RBC—Red blood cells (million/mm^3; WBC—white blood cells (1000/mm^3); Hb—haemoglobin (g/100 ml); N—neutrophils (%); L—lymphocytes (%); M—monocytes (%).

* Normal values for RBC 9–19; Hb 12.7–14.2 (Kolmer *et al.*, 1951).

Discussion

The present study has indicated the manner of organophosphorus poisoning in the domestic goat. Animals dosed with 0.5 mg/kg/day for 110 days showed severe signs of poisoning with highly significant inhibition of the acetylcholinesterase activity. It is of interest to observe here that guinea-pigs which were exposed to different doses of quinalphos did not show clinical signs of organic phosphorus poisoning up to 30 days. However the inhibition of acetylcholinesterase activity was highly significant both in guinea-pigs as well as in goats after quinalphos treatment (Dikshith *et al.*, 1980a). Similarly, while the guinea-pigs failed to show any morphological change in the vital organs, the liver of goats exposed to quinalphos for 110 days indicated mild cellular changes associated with biochemical alterations.

The activity of liver transaminases and protein showed a significant decrease in the quinalphos treated goats. In contrast to liver, the level of serum transaminases was unchanged. Absence of liver necrosis corelates with the unaltered levels of serum transaminases. However the activity of alkaline phosphatase in the serum increased significantly.

There was a highly significant decrease in RBC and WBC counts in the male goats after 110 days of treatment but were within the normal range of a healthy goat.

Experiments suggest that on stopping the quinalphos treatment followed by a period of rest, the decreased acetylcholinesterase activity as well as the lowered RBC and WBC counts returned to normal values. The pattern of recovery in these animals is similar to that observed on feeding the methyl demeton (Dikshith *et al.*, 1980c).

This study is of relevance since it suggests that occupational workers handling organophosphorous compounds as spray and formulations need periodical surveillance and rest. Such periodical check and reentry may minimise the incidence of poisoning.

Acknowledgements

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Effect of temperature on endocytosis and degradation of sulphated proteoglycans by cultured skin fibroblasts

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Abstract. Temperature up to 16°C reduced endocytosis of [^{35}S]-proteoglycans by human skin fibroblasts to less than 15% of that at 37°C. At temperatures between 20-26°C endocytosis was more than 50%. At temperatures below 26°C, the relative rate of degradation of endocytosed [^{35}S]-proteoglycans was several fold less than the rate of endocytosis.

Codistribution of endocytosed [^{35}S]-proteoglycans and the lysosomal marker enzyme β-hexosaminidase upon subcellular fractionation indicated that endocytotic vesicles containing [^{35}S]-proteoglycans had fused with lysosomes at 37°C and at 16°C. The prolonged half lives of endocytosed [^{35}S]-proteoglycans at 16-26°C could not be explained merely by a temperature dependent reduction of catalytic activity of lysosomal enzymes participating in the degradation of sulphated proteoglycans.

Keywords. Proteoglycan endocytosis; lysosomes; reduced degradation.

Introduction

Cells maintained in culture synthesize sulphated proteoglycans and distribute them in varying amounts into extracellular, cell surface associated and intracellular pools (Neufeld and Cantz, 1973; Kresse *et al.*, 1975). Previous studies have shown that arterial and skin fibroblasts internalize sulphated proteoglycans from the exterior through a process of adsorptive endocytosis involving specific binding of these macromolecules to cell surface receptors (Kresse *et al.*, 1975; Prinz *et al.*, 1978; Truppe and Kresse, 1978). The endocytotic vesicles so formed fuse with the lysosomes where the sulphated proteoglycans are rapidly degraded. Pastan and Willingham (1981) have recently described the formation of a new short lived subcellular organelle called "receptosome" which selectively delivers macromolecules entering cells via receptor mediated endocytosis through clathrin coated areas of the cell membrane (coated pits) to intracellular orgnelles such as lysosomes and the Golgi-endoplasmic-reticulum-lysosome (GERL) (Pastan and

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Abbreviations used: GERL, Golgi-endoplasmic-reticulum-lysosome; MEM, Eagle's minimum essential medium.

Willingham, 1981). It is not known at present whether sulphated proteoglycans are internalized via receptosomes. Absorptive endocytosis is inhibited at low temperature. This may result from deficiency of ATP (Jacques, 1969), inhibition of the recycling of receptors (Weigel, 1981) or impaired fusion of endocytotic vesicles with lysosomes (Dunn *et al.*, 1980).

In our earlier studies (Prinz *et al.*, 1978), the kinetics of proteoglycan binding to cell surface suggested the presence of high affinity binding sites on cell surface and the protein core of the proteoglycan was found to be essential for binding to cell surface. As an extension of these studies, the effect of temperature on the binding, internalization and degradation of the sulphated proteoglycans by human skin fibroblasts was examined.

Materials and methods

Sodium [^{35}S]-sulphate, carrier free (5 Ci/mg S) was obtained from Amersham Buchler, Braunschweig, Germany. Sephadex G-200 and Percoll were obtained from Pharmacia, Uppsala, Sweden.

Cell culture

Human skin fibroblasts from healthy individuals were maintained at 37°C in 5% CO_2 in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany), antibiotics and non-essential amino acids as described (Cantz *et al.*, 1972).

Preparation of [^{35}S]-proteoglycan from fibroblast secretions

Confluent cultures of human skin fibroblasts in 75 cm^2 Falcon plastic flasks were incubated in the presence of 15 ml serum free medium containing 7 µCi/ml [^{35}S]-sulphate for 5 days and [^{35}S]-proteoglycan secreted into the medium were purified by gel filtration as described earlier (Prinz *et al.*, 1978). The [^{35}S]-proteoglycans had a specific activity of 15×10^6 cpm/µmol uronic acid.

Measurement of [^{35}S]-proteoglycan endocytosis

Fibroblasts grown to confluency in 21 cm^2 Falcon plastic dishes were incubated in 21 cm^2 Falcon dishes in 1.5 ml medium containing [^{35}S]-proteoglycans at a concentration of 7.5 nmol disaccharides/ml. The dishes were maintained at different temperatures in air tight boxes under a gas phase of 5% CO_2 in air for 24 h. Cells were harvested by trypsinisation at the respective temperatures. Endocytosis, adsorption and degradation were determined earlier (Kresse *et al.*, 1975; Prinz *et al.*, 1978). The rate of endocytosis and degradation was independent of the amount of [^{35}S]-proteoglycans added as its concentration was very high.

Subcellular fractionation over Percoll

Confluent monolayer cultures in 75 cm^2 Flasks were maintained in 7 ml medium containing 7.5 nmol disaccharide/ml of [^{35}S]-proteoglycan for 24 h. Cells were harvested by trypsinisation at 16°C. All subsequent operations were done at 4°C. After washing with saline and then with MEM containing 10% calf serum, the cell pellet was washed with 0.25 M sucrose, resuspended in 0.25 M sucrose, kept for 10 min under nitrogen at 10 atm and homogenized with a glass homogenizer. The

homogenate was centrifuged at 600 *g* for 10 min at 4°C. The postnuclear fraction was applied on a Percoll gradient (starting density 1.07 g/ml) centrifuged in a vertical rotor VTi 50 (Beckman Instruments) at 20000 *g* for 1 h at 4°C (Pertoft *et al.*, 1978). Fractions of 2 ml were collected from the bottom by filling from the top with paraffin oil. In each fraction, density (Jurd and Rickwood, 1978), activity of β-hexosaminidase and radioactivity were determined.

Determination of half life of endocytosed [^{35}S]-proteoglycans

The rate of degradation of endocytosed [^{35}S]-proteoglycan was determined by measuring the rate of release of alcohol soluble radioactivity in a medium containing no radioactive material.

Other methods: β-Hexosaminidase, β-glucuronidase, α-N-acetyl-glycosaminidase and arylsulphatase B were determined as described (Von Figura, 1977). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Radioactivity was measured in a Packard liquid scintillation spectro-meter model 2450 B using Instagel (Packard Instruments) as scintillation medium.

Results and discussion

Effect of temperature on the endocytosis of [^{35}S]-proteoglycan

Below 16°C uptake was less than 10% of that at 37°C whereas at 20-26°C, 40-50% residual uptake was observed (figure 1). Between 16-26°C the rate of degradation, however, was 3-14% of that at 37°C. Thus in this range of temperature uptake was
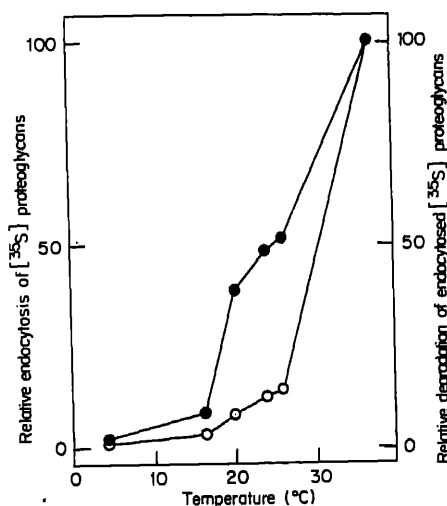
Figure 1. Endocytosis (●) and degradation (○) of [^{35}S]-proteoglycans by human skin fibroblasts as a function of temperature.

[^{35}S]-Proteoglycan endocytosis and degradation of endocytosed [^{35}S]-proteoglycans were determined after incubation of fibroblasts in the presence of [^{35}S]- proteoglycans for 24 h at temperatures between 16°C and 37°C. Endocytosis and degradation are expressed as % of the values at 37°C. At 37°C 12.8% of added [^{35}S]-proteoglycans were endocytosed and 65% of the endocytosed material had become degraded during the 24 h incubation period.

affected less than degradation of sulphated proteoglycans. The amount of proeoglycans absorbed to the cell surface measured as the amount released by trypsin was not affected by varying the temperature. It was 10% less at 16°C than at 37°C (data not given).

Subcellular fractionation after endocytosis at 16°C

A reduced rate of degradation at 16-26°C could result either from impairment of the fusion of endocytotic vesicles with lysosomes and/or from a decrease of the lysosomal activity at 16-26°C.

To decide whether the retarded rate of degradation was due to the inaccessibility of the endocytosed material to the lysosomes at this lower temperature, fibroblasts were incubated in the presence of [^{35}S]-proteoglycans at 16°C for 24 h and were
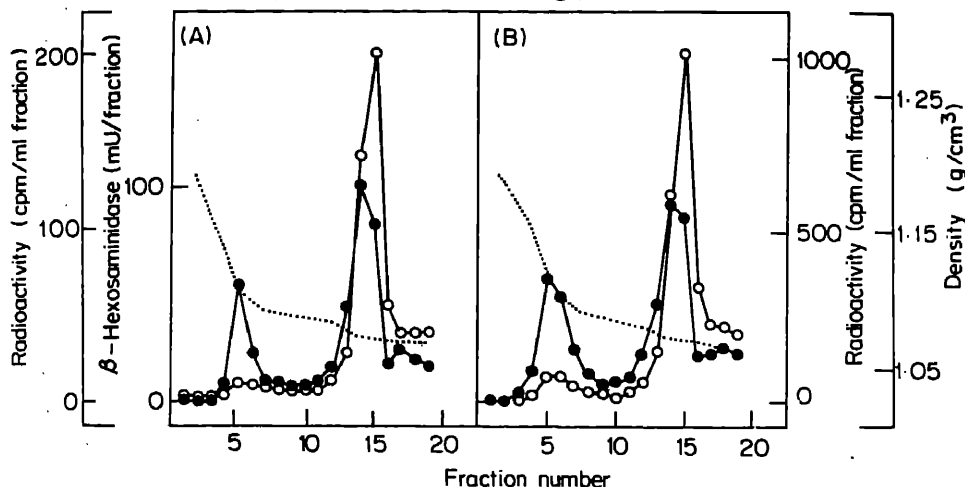
Figure 2. Subcellular fractionation on Percoll.

Postnuclear supernatant of skin fibroblasts which had been incubated in the presence of [^{35}S]-proteoglycans for 24 h at 16°C (A) or for another 160 min at 37°C (B) were fractioned on an isoosmotic gradient of Percoll. [^{35}S]-Radioactivity (●), β- hexosaminidase activity (O) and density (........) were determined in each fraction.

fractionated on an isoosmatic density gradient made of percoll. The cells incubated with [^{35}S]-proteoglycans for 24 h at 16°C and then for 160 min at 37°C to allow for transfer of internalized material into lysosomes served. as a control. A similar distribution pattern of [^{35}S]-glycosaminoglycans and β-hexosaminidase activity which served as a marker for lysosomes was obtained on fractionating the postnuclear supernatant over Percoll (figure 2) in both cases. The relative distribution of [^{35}S]-glycosaminoglycans and β-hexosaminidase among the earlier reported two classes of lysosomes, a dense fraction and a lighter density fraction (for discussions of these two classes of lysosomes see ref. Rome *et al.* (1979) was similar. The presence of [^{35}S]-glycosaminoglycans in fractions rich in lysosomes indicates that internalized proteoglycans do reach lysosomes at temperatures of 16-26°C. This conclusion is based on the assumption that fusion of endocytotic vesicles containing proteoglycan does not take place during the isolation and fractionation procedure. In hepatocytes, where endocytotic vesicles containing

asialofetuin do not fuse with lysosomes at 20°C or below (Dunn *et al.*, 1980), the endocytotic vesicles were stable.

Half life of endocytosed $[^{35}S]$-proteoglycan at low temperature

The endocytosed material was found to be degraded to inorganic $^{35}SO_4$ with a half life of 25 h at 37°C, whereas at 16°C it was degraded with a half life of 180 h (figure 3). At 16°C the rate of degradation is therefore about 7-fold reduced. Thus
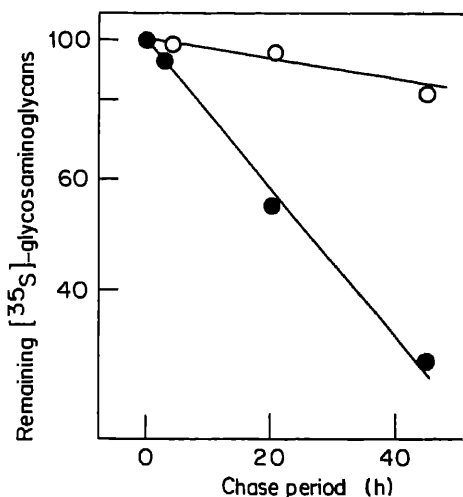
Figure 3. Half life of intracellular $[^{35}S]$-proteoglycans.

Fibroblasts were incubated in the presence of $[^{35}S]$-proteoglycans at 37°C (●) and at 16°C (○). After 24 h the medium was removed, the cell layer washed 5 times with proteoglycan free medium and subjected to chase for upto 42 h. The degradation of $[^{35}S]$-proteoglycans during the chase period was determined as described in "Materials and methods".

the relative accumulation of endocytosed proteoglycans at temperatures between 16-26°C appears to result from a decreased capacity of lysosomes to degrade proteoglycans at low temperature. The levels of lysosomal enzymes involved in degradation of proteoglycans, β-glucuronidase, α-N-acetyl-glucosaminidase, β-hexosaminidase and arylsulphatase B were not affected by growing fibroblasts for 24 h at 16°C. When catalytic activity of these enzymes were measured at 37, 26 and 16°C, respectively, a reduction of 50-60% in activity was observed at 26°C and compared to 37°C and of 70-85% at 16°C.

Our results show that sulphated proteoglycans endocytosed at low temperature accumulate intracellularly. In contrast to hepatocytes, the accumulation of endocytosed material is not due to impaired fusion of endocytotic vesicles with lysosomes, but to impaired degradation intralysosomally. The decreased rate of degradation at lower temperatures, notably at 26°C cannot fully be explained by the effect of temperature on the catalytic activity of degradative enzymes. The effect of temperature on the rate of degradation of proteoglycans in intact cells may also be related to the recent findings that degradation of proteoglycans in intact lysosomes is stimulated by ATP and acetyl-CoA (Rome and Crain, 1981).

Acknowledgement

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Studies on *Madhuca butyraceae* seed proteins

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Abstract. Defatted *Madhuca butyraceae* seeds contain 24% of crude protein and 10.4% of saponins. The solubility of *Madhuca* seed proteins was determined in water and NaCl as a function of pH and minimum solubility occurred at pH 4.0. The proteins consist of three components with $S_{20,w}$ values of 2.2, 9.8 and 15.4. On gel filtration the proteins gave three peaks and on diethylaminoethyl cellulose chromatography they resolved into two components. The *in vitro* digestibility of *Madhuca* seed protein was found to be 69% when assayed with a pepsin-pancreatin system.

Keywords. *Madhuca* proteins; physicochemical studies; *in vitro* digestibility.

Introduction

Madhuca butyraceae is grown in the Himalayan areas (such as Sikkim and Bhutan) and about 1.2 lakh tonnes of its seeds are estimated to be available annually. The flower of *Madhuca* are used as a source of alcohol. The seed contains considerable amount of fat, known as Phulwara butter (Annon, 1952) and is used in the treatment of rheumatism (Kirtikar and Basu, 1935). The defatted meal contains 25% protein (Mitra and Awasthi, 1962) and saponins (10-25%) which are toxic. The present investigation forms a part of an overall study of the chemical characteristics of defatted *Madhuca* seed flour and its possible use as a dietary constituent. The extractability and physico-chemical characteristics of the *Madhuca* seed proteins are presented here.

Materials and methods

The seeds and preparation of meal

Madhuca butyraceae seed kernels were obtained from the National Botanical Research Institute, Lucknow. Lipids were removed by crushing the kernels in a "Hander" crusher. The meal was then ground to 0.2 mm size and extracted at least six times with *n*-hexane to remove the residual fat. The defatted meal was powdered and passed through a 60 mesh sieve before use.

Chemical composition

Moisture, ash, crude fibre, fat and carbohydrates were estimated by standard AOAC methods (1975). Total and non-protein nitrogen were estimated by the Kjeldahl method (Pearson, 1970).

Extractability of proteins

Two g of defatted flour was suspended in 20 ml of the aqueous solvent and the slurry pH was adjusted to the desired value by adding 2 N NaOH or 2N HCl. The suspension was stirred for 1 h at room temperature (~28°C) and centrifuged at 5000 *g* for 20 min. The nitrogen content of the supernatant was estimated by the Kjeldahl method.

Since the extractability of the proteins is low in water and NaCl, a two stage repeated extraction procedure using 1 M NaCl (pH 8) was adopted. The supernatant was first concentrated and dialysed against the buffer solutions for 48 h.

Gel filtration

Sepharose-6B 100 was packed into a 1.5 × 100 cm column. The dialysed sample (3 ml) containing about 50 mg of protein was loaded on the column and the protein eluted with 0.025 M tris-glycine buffer of pH 8.3 containing 1 M NaCl. Fractions (3 ml) were collected and the absorbance measured at 280 nm.

DEAE-cellulose chromatogrphy

Diethylaminoethyl-(DEAE)-cellulose after regeneration (Peterson, 1970) was packed into a 2 × 22 cm column under pressure and equilibrated with 0.02 M sodium phosphate buffer of pH 7.6. About 50 mg of the protein was loaded on the column. It was then eluted with a linear gradient of 0.0 − 0.8 M NaCl. Three ml fractions were collected and the absorbance measured at 280 nm. The concentration of NaCl was estimated as described by Rieman *et al.* (1951).

Sedimentation velocity experiment

The experiment was performed using 1% protein solution in 0.1 M phosphate buffer of pH 7.8 containing 1 M NaCl at room temperature (~28°C) at 56,100 rpm in a Spinco Model E Analytical Ultracentrifuge equipped with a rotor temperature indicator unit and phase plate schlieren optics. $S_{20,w}$ was calculated by the standard procedure (Schachmann, 1959).

Polyacrylamide gel electrophoresis

Polyacrylamide gels (7.5%) in 0.01 M tris-glycine buffer of pH 8.3 were prepared by the standard procedure. About 100 μg of the protein was loaded on each gel in tubes of 0.5 × 7.5 cm and the electrophoresis was carried out for 1 h at 3 mA/tube. The protein components on the gels were then identified by staining with 0.5% Amido Black for 1 h followed by destaining in 7.5% acetic acid. The gels were then scanned in a Joyce Lobel scanner.

In vitro digestibility

In vitro digestibility was determined by the method of Akeson and Stahmann (1964). The protein was incubated with pepsin for 3 h, followed by pancreatin for a total period of 24 h at 37°C. The reaction was arrested by 10% trichloroacetic acid

and the nitrogen content was determined by the microKjeldahl procedure. The estimations were made at 4 h intervals.

Results and discussion

The chemical composition of the defatted meal is given in table 1. Defatted meal contains 24% protein. A comparable value has been reported by Mitra and Awasthi

Table 1. Chemical composition of defatted *Madhuca* flour (g%).

Moisture	12.0
Total protein (N×6.25)	24.0
Lipids	1.0
Non-protein Nitrogen	0.5
Saponins	10.4
Total carbohydrates	34.0
Ash	6.0
Crude fibre	8.0

(1962). The solubility profile of *Madhuca* seed protein in water shows a "U" shaped pattern (figure 1), indicating only one solubility minimum which is characteristic of most plant proteins (Fontaine *et al.*, 1944; Smith and Circle,1938).
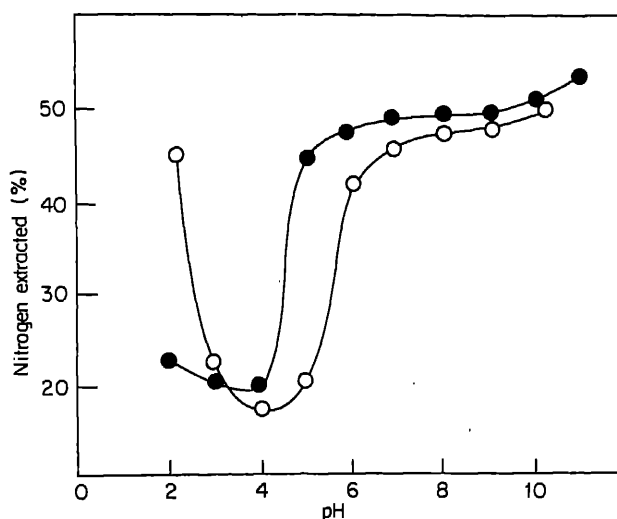
Figure 1. Extractability of *Madhuca* seed as a function of pH. Water O); 1 M NaCl (●).

In water, the minimum solubility was found to be at pH 4. At this point, about 16% of the total nitrogen was found to be soluble, whereas at pH 8, about 50% of the nitrogen was solubilized. This appears distinctly different from most plant proteins which show 80-90% solubility at pH 8 (Fontaine *et al.*, 1944, Smith and Circle, 1938). The lower extractibility of *Madhuca* proteins may be due to the presence of saponin-protein complexes. The solubility in 1 M NaCl was slightly higher than in water at pH 8, but the minimum solubility pH was not significantly altered, with only 20% of the nitrogen remaining soluble.

On gel filtration, the proteins were separated into three fractions (figure 2) with V_e/V_o values of 0.97 (I), 2.3 (II) and 3.13 (III) respectively. The first fraction was turbid and eluted near the void volume.
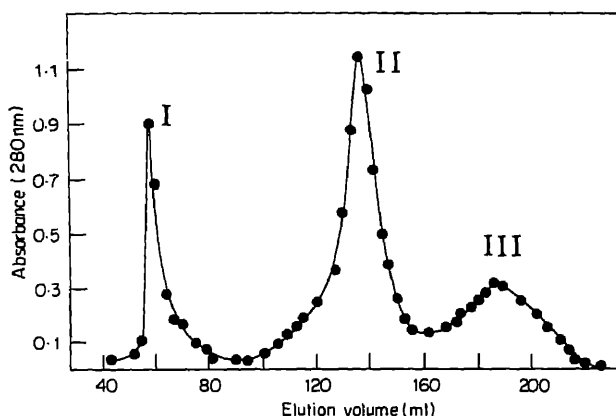
Figure 2. Gel filtration pattern of *Madhuca* seed proteins on Sepharose-6B-100.

The total proteins were fractionated into two components on DEAE-cellulose chromatography (figure 3) (0.0 − 0.8 M NaCl gradient). One fraction was eluted unadsorbed while the other eluted at 0.29 M NaCl concentration.
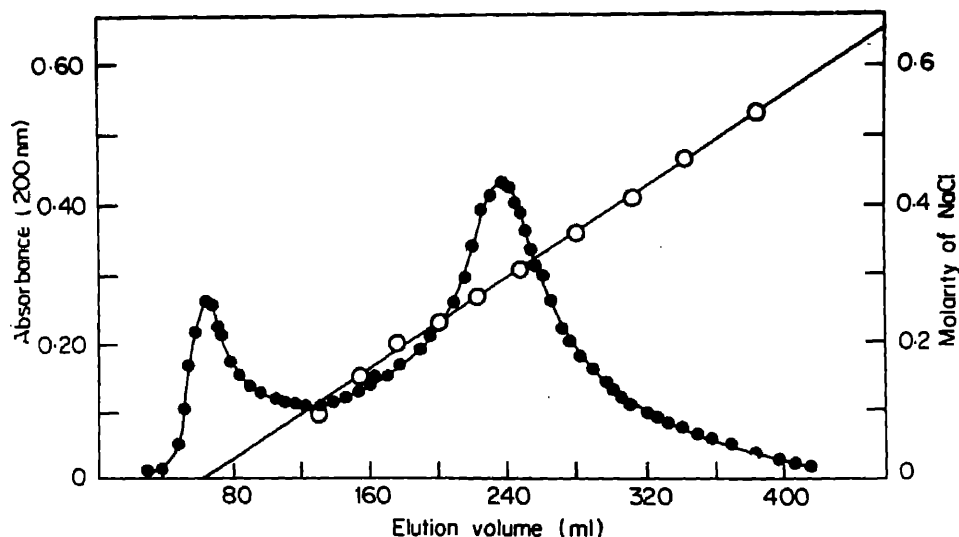
Figure 3. DEAE-cellulose ion-exchange chromatographic pattern of *Madhuca* seed proteins.

The sedimentation velocity pattern of the total proteins showed the presence of three peaks having $S_{20,w}$ values of 2.2, 9.8 and 15.4 (figure 4). The relative proportions of the three fractions were 75.8%, 21.3% and 2.9% respectively.

4

Figure 4. Sedimentation velocity pattern of *Madhuca* seed proteins. Sedimentation proceeds from left to right.

Gel electrophoresis of the total proteins in 0.01 M tris-glycine buffer showed six bands (figure 5).Two of them had higher mobility. The major fraction contributes about 30% of the proteins as read on microdensitometric scanning.
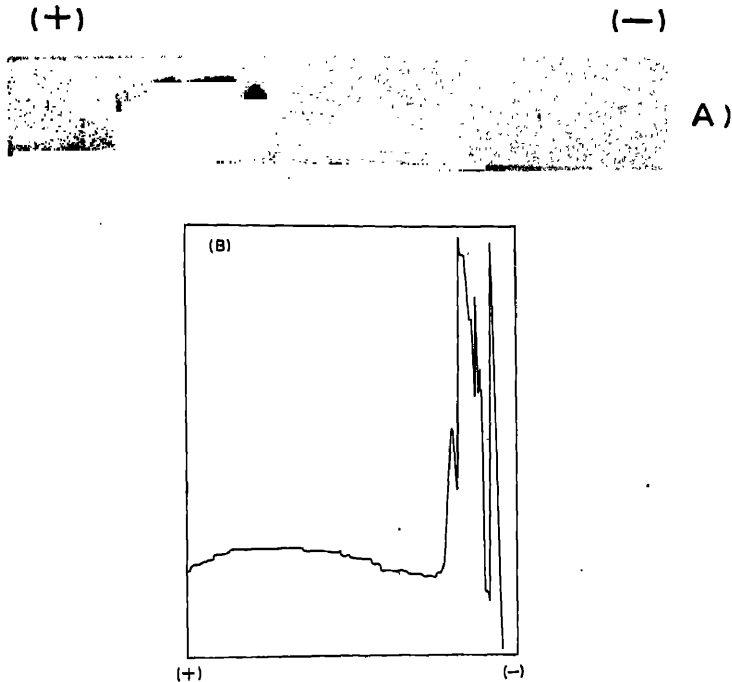
figure 5. Polyacrylamide gel electrophoretic pattern of *Madhuca* seed proteins A. Gel pattern B. Microdensitometric scanning of the gel.

In-the pepsin-pancreatin system, the initial digestibility was ~30% due to non-protein components. The protein hydrolysis reached a maximum level (69%) at 11 h (figure 6). After this, there was no further increase in digestibility.
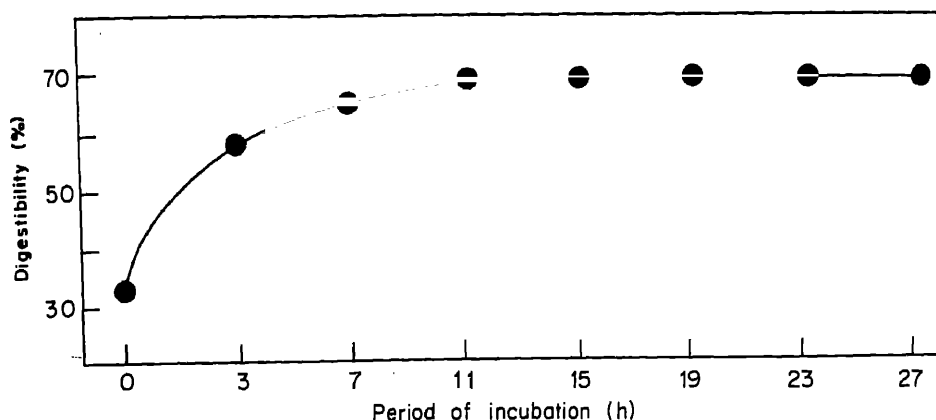
Figure 6. *In vitro* digestibility of *Madhuca* protein.

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Influence of red pepper and capsaicin on body composition and lipogenesis in rats

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Abstract. Inclusion of red pepper or its active principle 'capsaicin' in the diet led to a lowering of total lipids, particularly triglycerides in the liver. The total body fat was lowered in animals fed red pepper or capsaicin but not in animals fed paprika powder which had negligible capsaicin content. Hyperlipogensis and hypertriglyceridemia caused by fructose feeding were significantly were decreased in capsaicin-fed animals. Activities of the key lipogenic enzymes were reduced as reflected by decreased lipogenesis.

Keywords. Red pepper; capsaicin; lipogenesis.

Introduction

Earlier studies showed that red pepper or its active principle 'capsaicin' partly counteracted the accumulation of fat in the liver of rats fed high-fat or choline-deficient diets (Sambaiah *et al.*, 1978) and in ethionine or carbon tetrachloride induced fatty livers (unpublished work).

The effect of capsaicin may be due to (a) its reduction of hepatic lipogenesis; (b) enhanced transport of lipids to serum or (c) increased oxidation of lipids in the tissues. This communication describes findings about the influence of red pepper and capsaicin on total body fat and lipogenic enzyme activities in the liver of rats.

Materials and methods

Red pepper (*Capsicum frutescens*) was obtained locally and ground to 30 mesh size. Paprika (*Capsicum annuum*) powder was obtained as a gift from Lalocsa Paprika Co., Hungary. Capsaicin content in these samples was determined (Govindarajan and Ananthakrishna, 1974) to be 0.3 and 0.005% respectively. Male rats of Wistar strain weighing about 200 g were used in all the experiments. The basal diet fed to rats consisted of 18% protein (casein), 10% sucrose, 10% fat (groundnut oil), 59% corn starch, 2% salt mixture (Hubbel *et al.*, 1937) and 1% vitaminised starch (Chapman *et al.*, 1959). In addition, rats daily received adequate quantities of vitamins A, D and E. Experimental diets were prepared by incorporating 5% red pepper, 5% paprika powder or 15 mg% capsaicin (Fluka AG, Switzerland) in the basal diet in place of corn starch. At the end of the feeding period, the rats were sacrificed and various tissues collected for analysis.

Body composition

For determining body composition the weighed carcass (free from blood, liver and the intestinal contents) was cut into small pieces and dried to constant weight at 100°C. The difference in weight denoted the water content. The dried carcass was used for the estimation of fat after extraction by gravimetry (Folch *et al.*, 1957), protein by micro-Kjeldahl (nitrogen content $\times$ 6.25) and ash by the A.O.A.C. procedure (A.O.A.C., 1975).

Fructose induced hypertriglyceridemia

Two groups of animals were fed either the basal or experimental diet containing 15 mg% capsaicin, for 5 weeks. At the end of the feeding period, in addition to the diet both groups of rats received 10% fructose solution instead of drinking water for a period of 7 days.

Lipid analysis

Total lipids in liver and serum were extracted and purified according to Folch *et al.* (1957) and estimated by gravimetry. Cholesterol, phospholipids and triglycerides were determined using methods described by Searcy and Bergquist (1960), Marinetti (1962) and Fletcher (1968) respectively.

Enzyme assays

Rats were killed by decapitation and the livers were removed quickly and washed with cold 0.15 M KCl. About 1 g of liver tissue was homogenised in cold 0.15 M KCl and the homogenate was centrifuged at 1000 g at 0-4°C for 15 min ina Sorval RC 2B centrifuge. The supernatant was recentrifuged 100000 g for 1 h in a Beckman L2-65B ultracentrifuge and the resulting supernatant was used· for enzyme assays. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed by the method of Glock and McLean (1953) with modifications as described by Leveille (1972). Malic enzyme (EC 1.1.1.40) and citrate cleavage enzyme (EC 4.1.3.8) were assayed as described by Ochoa (1955), Cottom and Srere (1964) with modifications as suggested by Yeh *et al.* (1970). The protein content of homogenates was determined by the method of Lowry *et al.* (1953). The enzyme activities were expressed as units/mg protein and a unit is defined as the amount of enzyme which catalyzes the utilization of one nmol of substrate per min.

Statistical analysis

Data were analyzed by Student 't' test and levels of significance denoted in tables by the letters a-p $<$ 0.05, b-p $<$ 0.02, c-p $<$ 0.01, d-p $<$ 0.002 and e-p $<$ 0.001 (Snedecor and Cochran, 1967).

Results

Influence of red pepper/capsaicin on body composition

The inclusion of capsaicin in the diet significantly reduced the weight of liver compared to the controls, whereas red pepper and paprika had no such effect (table 1). Total lipid levels were reduced only in rats receiving red pepper or

capsaicin containing diets. The triglyceride component of the total lipids was lowered significantly, without any change in the phospholipid or cholesterol content. Supplementation of the paprika containing diet with capsaicin at 15 mg% brought about similar changes (table 1).

Table 1.   Effect of red pepper, capsaicin and paprika on liver lipids.

Parameters	Additions to basal diet				
	Nil	5% red pepper	15 mg% capsaicin	5% paprika	5% paprika ± 15 mg% capsaicin
Initial body weight (g)	208±3.34	207±2.78	208±3.40	208±3.25	208±2.98
Final body weight (g)	282±10.6	285±9.9	268±6.9	281±6.3	270±5.8
Weight of liver (g)	9.5±0.5	8.9±0.2	8.2^a±0.2	8.6±0.3	8.0^a±0.2
Liver lipids (mg/g)					
Total lipids	51.7±2.1	42.7^c±2.1	42.7^a±1.6	46.2±3.3	43.4^a±0.8
Triglycerides	9.1±0.8	5.5^c±0.8	5.6^a±1.1	6.8±1.0	3.19^c±0.3
Pospholipids	26.7±0.8	27.5±1.1	27.6±1.3	26.4±0.5	27.2±0.6
Cholesterol	3.7±0.1	4.0±0.1	3.5±0.1	4.1±0.1	4.1±0.2

Values are mean ±SEM for 5 rats fed for 8 weeks

The carcass composition of rats feed different experimental diets is presented in table 2. A significant decrease in total body lipid with a concomitant increase in moisture was found in rats fed pepper or capsaicin. Paprika powder in contrast with red pepper, did not alter the body fat content. There were no differences observed in the protein and ash content among rats fed the different diets.

Fructose induced hypotriglyceridemia

Triglycerides in the liver of rats receiving diet containing 15 mg% capsaicin, were significantly reduced. Total lipids, phospholipids and cholesterol levels were not altered (table 3). A significant reduction was also observed in serum triglyceride levels of rats fed capsaicin containing diet (table 3).

Effect of capsaicin on some lipogenic enzyme activities in liver

It is seen (table 4) that all the four key lipogenic enzyme activities were significantly decreased though to different degrees. Of the three NADPH producing enzyme reactions, glucose-6-phosphate dehydrogenase showed the maximum decrease. Feeding of capsaicin caused a significant decrease in the activity of citrate cleavage enzyme also.

Table 2. Effect of red pepper, capsaicin and paprika on carcass composition in adult rats.

Parameters	Additions to basal diet				
	Nil	5% red pepper	15 mg% capsaicin	5% paprika	5% paprika ± 15 mg% capsaicin
Corrected* body weight (g)	253±8.8	256±9.1	241±5.9	255±5.9	242±5.8
Carcass composition					
% of corrected body weight					
Water	57.3±1.7	64.9*a*±1.3	65.4*a*±1.8	60.6±0.8	63.8±1.7
Protein	14.3±0.6	14.3±0.4	14.1±0.3	14.4±0.4	14.4±0.5
Ash	3.1±0.1	3.3±0.1	3.1±0.1	3.1±0.1	3.1±0.2
Lipid					
% of corrected body weight	25.3±2.4	17.5*a*±1.5	17.4*a*±2.1	21.9±0.7	18.7*a*±1.4
% of dry carcass	52.1±3.0	41.9*a*±2.7	41.2*a*±2.5	44.7±2.7	40.3*a*±2.5

Values are mean ±SEM for 5 rats fed for 8 weeks

* Corrected body weight represents body weight minus liver blood and intestinal contents. Carcas composition on fat-free basis: water, 66—70%; protein, 17-20%, ash, 9.3-10%.

Table 3. Influence of capsaicin on lipid levels in liver and serum in fructose induced hypertriglyceridemia.

Treatment	Additions to basal diet		
	Nil	Nil	15 mg% capsaicin
	Nil	Fructose* feeding	Fructose feeding
Liver lipids (mg/g)			
Total	46.3±1.8	51.4±2.5	48.1±2.1
Triglycerides	10.6±0.7	15.3±1.2	6.2±1.5*c*
Phospholipids	23.8±3.4	19.6±0.9	19.7±0.9
Cholesterol	3.6±0.1	7.4±0.4	8.4±0.6
Serum triglycerides	99.5±19.2	190.5±24.6	121.7±16.2*a*

Values are mean ±SEM 8 rats fed for 6 weeks

* Rats were given drinking water containing 10% fructose for 7 days before they were sacrificed.

Table 4. Effect of capsaicin on some liver lipogenic enzymes*

	Addition to basal diet	
	Nil	15 mg% capsaicin
Glucose-6-phosphate dehydrogenase	57.8±3.3 (6)	31.4±3.9c (6)
6-Phosphogluconate dehydrogenase	33.6±1.6 (7)	26.5±2.8a (7)
Malic enzyme	72.6±4.4 (9)	45.8±7.1a (6)
Citrate cleavage enzyme	16.1±0.5 (6)	11.7±0.9d (6)

Values are mean ± SEM with number of rats in parenthesis in each group. Period of feeding 6 weeks.

* Enzyme activity is expressed as units where one unit of enzyme is one nanomol of substrate utilized/minute/mg protein.

Discussion

The present investigations have shown that both red pepper and capsaicin feeding significantly counteract the increase in triglyceride levels in livers of rats fed normal and fructose containing diets. A decrease in serum triglyceride levels is also brought about by capsaicin feeding in fructose fed animals (tables 1 and 3).

Fructose is known to enhance hepatic lipogenesis (Zakim *et al.*, 1967; Sullivan *et al.*, 1971) and cause hypertriglyceridemia (Bar-On and Stein, 1968; Hill, 1970). The former condition is likely to be brought about by an increase in lipogenic enzyme activities (Romsos and Leveille, 1974) observed in the livers of rats fed fructose rich diets.

Fatty acid synthesis involves the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and citrate cleavage enzyme, to various degrees. A reduction of these enzymes leads to decreased availability of reduced nucleotides as well as extra mitochondrial acetyl CoA both of which are required for lipogenesis. A good correlation between pentose phosphate pathway and the rate of fatty acid synthesis in rat adipose tissue was demonstrated earlier (Kather *et al.*, 1972). Malic enzyme also participates in the generation of extramitochondrial NADPH which supplements the requirement of reduced equivalents to fatty acid synthesis. The citrate cleavage enzyme is generally accepted to be of major importance in supplying the needed acetyl COA for lipogenesis (Greville, 1969). Capsaicin probably lowers lipogenesis as reflected by the reduced activity of the key lipogenic enzymes (table 4).

Analysis of the body composition of adult rats fed either red pepper or capsaicin in the diet revealed a significant reduction in the total body lipid levels with no change in total body protein. The reduced level of fat in the body is significant both when the fat is expressed as per cent of corrected body weight or as per cent of dry carcass (table 2). In contrast to the reduction of fat, the change in body water was not real. Thus, although there was a significant increase in water content in the bodies of animals fed red pepper or capsaicin, this disappeared when the water content of the carcass was expressed on fat-free basis. These results substantiate the findings of the effect of capsaicin on fatty acid synthesis as indicated by the enzyme activities.

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Regulation of phosphoglycerate mutase in developing forespores and dormant spores of *Bacillus megaterium* by the *in vivo* levels of phosphoglycerate mutase inhibitor

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Abstract. *Bacillus megaterium* accumulated 3-phosphoglycerate during sporulation which was utilized during spore germination. During sporulation a protein was synthesized before or at the start of 3-phosphoglycerate accumulation inside the developing spores about 1.5 h before dipicolinic acid accumulation. This protein has an affinity for Mn^{2+} and other divalent metal ions and inhibits phosphoglycerate mutase activity which has been shown to require Mn^{2+} However, the levels of the inhibitor decreased considerably (75-85%) during spore germination. No appreciable amount of the inhibitor was detected in the vegetable cell and mother cell compartment; however, the forespore compartment possesses an activity comparable to that of dormant spores. The partially purified inhibitor has a molecular weight of 11,000 and possesses both high and low affinity binding sites for Mn^{2+} and Ca^{2+} as determined by Scatchard plot analysis.

Keywords. *Bacillus megaterium*; 3-phosphoglyceric acid; phosphoglycerate mutase; mutase inhibitor; dipicolinic acid.

Introduction

Dormant spores of various Bacillus species contain an appreciable amount of 3-phosphoglyceric acid (3-PGA) which is accumulated late at a time in sporulation only within the developing forespores (Nelson and Kornberg, 1970; Setlow and Kornberg, 1970a, b; Singh *et al.*, 1977). The 3-PGA depot is stable in the forespore and dormant spore despite the presence of enzymes capable of catabolizing it, namely phosphoglycerate mutase (EC 2.7.5.3) enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40) at levels similar to those in growing cells and germinated spores (Singh *et al.*, 1977). Since 3-PGA accumulates in forespores even though the enzymes for 3-PGA catabolism are detected in extracts, at least one of these enzymes must have very little activity *in vivo* and become activated upon spore germination.

Abbreviations used: 3-PGA, 3-phosphoglyceric acid; 2-PGA; 2-phosphoglyceric acid; DPA, dipicolinic acid; SNB, supplemented nutrient broth; DEAE, diethylamino ethyl.

Previous work has implicated phosphoglycerate mutase as a key enzyme in the regulation of 3-PGA accumulation during sporulation (Singh and Setlow, 1978a, and 1979b). In this communication data are presented to show that phosphoglycerate mutase is the enzyme which is regulated to allow the accumulation of 3-PGA during sporulation followed by its utilization during spore germination. The regulation of this enzyme *in vivo* appears to be mediated, at least in part by the levels of free Mn^{2+}. The levels of free Mn^{2+} are likely to be controlled by the phosphoglycerate mutase inhibitor which is synthesised during sporulation and degraded upon spore germination, thereby relieving the inhibition of phosphoglycerate mutase.

Materials and methods

Chemicals and enzymes

Diethylaminoethyl—(DEAE)—cellulose and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were supplied by Sigma Chemical Co., St. Louis, Missouri, USA. Phosphoglycerate mutase and enolase were purified from log phase cells of *B. megaterium* as described by Singh and Setlow (1978b, 1979a). ADP was freed of contaminating ATP by incubating with hexokinase (EC 2.7.1.1) and glucose and subsequent boiling.

Organism, cultural conditions and isolation of forespores

All work described in this manuscript was carried out with *B. megaterium* QMB 1551, originally obtained from H.S. Levinson, U.S. Army Natick Laboratories, Natick, Massachusetts, USA. The organism was grown in a supplemented nutrient broth at 30°C, allowed to sporulate and were cleaned as described by Setlow and Kornberg (1969). The forespores were prepared as described earlier (Singh *et al.*, 1977).

Isolation and purification of phosphoglycerate mutase inhibitor

Lyophilized dormant spores were disrupted in dry state using Dental Amalgator (Wig-L-Bug) with acid cleaned sand as the abrasive (Sacks and Bailey, 1963). The dry powder was extracted for 1 h with ice-cold buffer A Tris (hydroxymethyl) aminomethane (Tris) — HCl, 50 mM; EDTA, 25 mM; pH 7.4 followed by centrifugation at 10,000 g for 20 min. The supernatant was discarded and the pellet washed thrice with Tris-HCl, pH 7.4, 50 mM. The residual material was dissolved in 50 mM Tris buffer pH 7.4 containing 5 mM $CaCl_2$ followed by incubation overnight at 37°C. The mixture was centrifuged and the pellet was reincubated as described above. Supernatants from both the preparations were pooled and dialysed overnight aganst Tris-buffer (50 mM) with three changes. The other steps followed in the purification of mutase inhibitor are listed in table 1. Mutase inhibitor was also isolated from forespores, germinated spores and the vegetative cells.

Assay of 3-PGA, phosphoglycerate mutase and mutase inhibitor

3-PGA was determined using the luciferase assay method (Setlow and Kornberg,

1970b) after conversion of 3-PGA to ATP using ADP plus enolase, phosphoglycerate mutase and pyruvate kinase. Samples of forespore or dormant spore extracts containing up to 10 n mol· of 3-PGA were incubated in a volume of 0.5 ml containing 50 mM Tris-HCL (pH 7.4); 100 mM, KCl; 5 mM MgSO₄; 300 μM ADP; 20 μg of rabit muscle pyruvate kinase, 18 μg of homogenous enolase prepared by the method of Singh and Setlow (1978) and 25 μg of purified phosphoglycerate mutase from *B. megaterium* prepared by the method of Singh and Setlow (1979a). The latter enzyme was free of adenylate kinase. After incubation of 5 min at 37°C, the reaction mixture was boiled for 5 min and an aliquot assayed for ATP. A control was run to correct for ATP contamination in ADP (<0.2%).

Assays of phosphoglycerate mutase utilized the discontinuous assay procedure described previously (Czok, 1974) where the first incubation containined only enzyme plus 3-PGA. After boiling, a second incubation was used to determine the amount of 2-PGA formed in the first incubation. The mutase inhibitor was assayed by adding in the first incubation mixture. The amount of mutase inhibitor which gives 50% inhibition of phosphoglycerate mutase is defined as one unit of inhibitor, units/mg of protein is called as specific activity.

Extraction of 3-PGA and DPA from forespores and dormant spores

Forespores were isolated at designated times (arrows in figure 1) as described earlier (Singh *et al.*, 1977) and spores prepared by growing *B. megaterium* in supplemented nutrient broth (SNB) medium. Spore preparation was free of vegetative cells and cell debris and contained >95% refractile forms when viewed in a phase contrast microscope (Setlow and Kornberg, 1969). 3-PGA and DPA were extracted from freeze dried forespores and dormant spores by boiling in 80% 1-propanol (Setlow and Kornberg, 1970a).

Analytical assays

DPA was assayed by the method of Rotman and Fields (1967). Protein was determined by following the procedure of Lowry *et al.* (1951). Growth of the organism was monitored by measuring absorbance at 600 nm with Spectronic-20 (Bausch and Lomb) spectrophotometer. Molecular weight of the mutase inhibitor was determined by methods published earlier (Whitaker, 1963; Weber and Osborne, 1969).

Binding efficiency of mutase inhibitor

Binding efficiency of the mutase inhibitor was determined by the equilibrium dialysis method (Englund *et al.*, 1969) except that the diameter and depth of the chambers was about 200 μl. The dialysis membrane used was Visking 20/32 type which had been boiled for 5 min in 5% Na₂CO₃ − 50 mM EDTA washed thrice with distilled water and stored in 50% ethanol at 4°C. Before use, membrane was hydrated in distilled water and blotted dry. To assemble the cells, approximately circular pieces of membrane about 0.5 inch diameter were placed between the two chambers in each set and the cells were then clamped into metal holders. Using

Hamilton syringe, 100 µl of mutase inhibitor, 25 mM Tris buffer pH 7.4 and 100 mM NaCl were placed in the chamber on one side of the membrane and 100 µl of distilled water, 25 mM Tris buffer pH 7.4, 100 mM NaCl and $MnCl_2$ (varying concentrations) containing 5 µl [54]Mn (5000 cpm) was placed on the other side of the membrane. The entire apparatus was shaken (25 rpm) at room temperature. After equilibration of the dialysis content (about 2 h), cell samples were removed from each side of the membrane with 10 µl Hamilton syringe for determination of radioactivity and binding efficiency of mutase inhibitor was calculated (Scatchard, 1949).

Calculation of binding data

Data were plotted according to the rearrangement of the equation for binding of the ligand (Mn^{2+}) to a mutase inhibitor as described by Setlow and Mansour (1972).

$$\frac{\bar{n}}{c} = \frac{1}{K_D}(\bar{n}-n)$$

where c is the free Mn^{2+} concentration and $\bar{n}$ is the average moles of Mn^{2+} bound/ unit of mutase inhibitor. Plots of $\bar{n}/c$ vs $\bar{n}$, yield a straight line which extrapolates to the abscissa at n, the number of identical, independent binding sites on the mutase inhibitor. The dissociation constant (K_D) was obtained from the slope of the line. The amount of Mn^{2+} bound to the inhibitor was determined from the difference in radioactivity between the two sides of the dialysis chamber. The radioactivity on the side lacking the enzyme inhibitor was used to calculate the concentration of the free ligand (Mn^{2+}).

Results

Accumulation of 3-PGA and mutase inhibitor

The organism grown in SNB medium accumulated 3-PGA (figure 1). Appearance
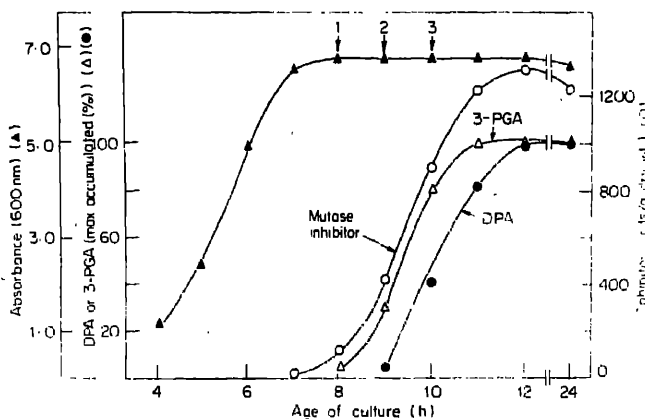
Figure 1. Accumulation of 3-phosphoglycerate acid, dipicolinate and mutase inhibitor during sporulation of *B. megaterium*. The organism was grown in supplemented nutrient broth and forespores were isolated at designated times as shown by arrows. 3-PGA, DPA and mutase were assayed.

of mutase inhibitor is quite interesting as it is likely to suppress the phosphoglycerate mutase activity *in vivo*. This possibly explained the accumulation of 3-PGA even in the presence of phosphoglycerate mutase, which was shown to require Mn^{2+} as a cofactor. Accumulation of DPA seemed to play no significant role in regulating phosphoglycerate mutase activity in the developing forespores because it starts about 1.5 h after 3-PGA accumulation.

The levels of mutase inhibitor and 3-PGA vary with the stages of the growth (table 1). As expected no appreciable amount of mutase inhibitor and 3-PGA were detected in the vegetative cells and germinated spores under normal conditions. The levels of 3-PGA remained constant when spores were germinated in a medium containing NaF (Singh and Setlow, 1979b). Parallel observations have

Table 1. Levels of phosphoglycerate mutase inhibitor and 3-phosphoglyceric acid at different stages of development of *B. megaterium*.

Stage of growth	Inhibitor (Units/g dry wt.)	3-Phosphoglycerate (μmol/g dry wt.)
Dormant spores	1275	21.6
Germinated spores	69	1.2
Vegetative cells	Undetectable	4.2
Forespores[a]:		
Sample 1	155	11.5
Sample 2	490	17.2
Sample 3	900	20.1

[a] Forespores isolated from cultures withdrawn at the time designated by arrows in figure 1 and prepared according to the methods described in materials and methods section.

also been reported with the vegetative cells of *B. subtilis* when grown in a Mn^{2+} depleted medium (Oh and Freese, 1976).

Purification of mutase inhibitor

The mutase inhibitor was isolated from dormant spores and the purification steps followed are shown in table 2. DEAE-cellulose elution profile (figure 2) of mutase

Table 2. Purification of phosphoglycerate mutase inhibitor from dormant spores of *B. megaterium*.

Purification steps	Total units	Units/mg protein	Recovery (%)	Fold Purification
Spore extract	3644	10.5	100	1
Heat treatment	3500	197.0	96	18.8
DEAE cellulose	2032	960.2	55.7	91.4
Sephadex G-100	1019	1203.6	27	114.6

Phosphoglycerate mutase — inhibitor was assayed as mentioned in the materials and methods section.

inhibitor was obtained after eluting the column (2.5 × 25 cm) with 50 mM Tris buffer pH 7.4 (100 ml each) containing NaCl (0 to 0.5 M) in a linear gradient with a
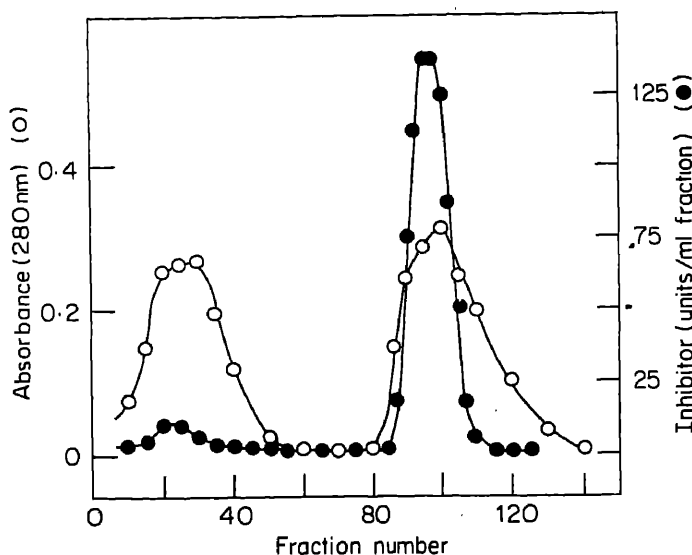
Figure 2. DEAE-cellulose chromatography of phophoglvcerate mutase inhibitor from dormant spores of *B. megaterium*. The spore extract (table 2, step 1) was dialysed overnight at 4°C against three changes of 50 mM Tris pH 7.4. The dialysed preparation was applied to a DEAE-cellulose column and eluted with a linear gradient from 0 to 0.5 M NaCl in 0.50 mM Trisbuffer pH 7.4).

flow rate of 25 ml/h. The fractions containing more than 50 units/ml were pooled and dialysed against 50 mM Tris buffer pH 7.4, concentrated using Pasteur pipette and dialysed again to remove salt. This concentrated preparation containing protein (50 mg) was layered on Sephadex G-100 column (2.5 × 72 cm) and eluted with Tris buffer (50 mM, pH 7.4) containing 100 mM NaCl with a flow rate of 5 ml/ h. This preparation was used for binding experiments and molecular weight determination.

Binding studies of mutase inhibitor

Binding properties of the mutase inhibitor with respect to Mn^{2+} and Ca^{2+} were done using the equilibrium dialysis technique. Results presented in table 3 show a

Table 3. Determination of binding constants of phosphoglycerate mutase inhibitor.

Addition	High affinity sites		Low affinity sites	
	K_D (μM)	n (nmol/ unit)	K_D (μM)	n (nmol/ unit)
$MnCl_2$ (3 mM)	0.35	0.29	47	1.82
$CaCl_2$ (2 mM)	1.2	0.47	60	2.32

dissociation constant of 47 μM for Mn2+, suggesting that the binding of Mn2+ was not strong. Similar results were obtained when Ca2+ was substituted in place of Mn2+ (unpublished results). This suggestion was further strengthened with the exchange experiments using cold Mn2+ or Ca2+ as the case may be (unpublished data).
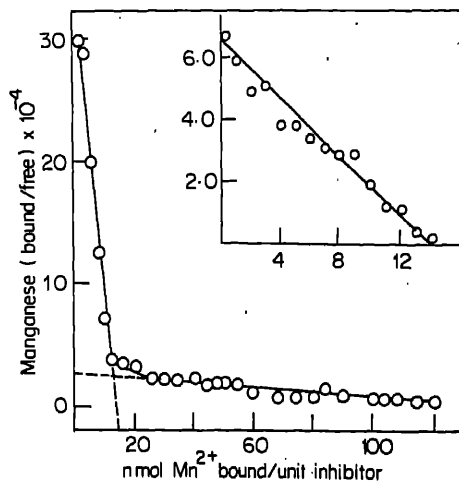
Figure 3. Manganese binding efficiency of mutase inhibitor. Binding studies were done by Scatchard plot analysis and binding constants, low and high affinity binding sites were calculated.

Scatchard plot analysis revealed the existence of low and high affinity binding sites which were determined by the extrapolation of lines on X and Y axis (figure 3). Table 3 shows the number of high and low affinity binding sites.

Discussion

3-Phosphoglycerate accumulated during the later stages of sporulation of *B. megaterium* i.e. about 1.5 h before the accumulation of DPA (figure 1). Previously, Singh *et al.* (1977) have shown that most (>95%) of the 3-PGA was utilized during spore germination to produce ATP needed for other metabolic processes. It was also suggested that the enzymes of 3-PGA catabolism namely, phosphoglycerate mutase, enolase and pyruvate kinase are present in the various stages of growth with no detectable changes in specific activities when tested *in vitro*. Therefore, it is reasonable to presume that these enzymes act rapidly *in vivo* in the first minutes of spore germination. However, at least one of these enzymes must have little or no activity within the forespores or dormant spores in which a large stable pool of 3-PGA is accumulated. A reason which is often given for the absence of enzyme activity in dormant spores is their low water content (Gould, 1977; Gould and Dring, 1975). This may be true in the dormant spores, but is highly unlikely during 3-PGA accumulation in the forespores since 3-PGA accumulation is followed by other events like DPA accumulation which should require a hydrated spore core. Consequently, a different mechanism seems necessary to explain regulation of

enzymes of 3-PGA accumulation. Phosphoglycerate mutase has been shown to require Mn^{2+} for its activity (Watabe and Freese, 1979; Singh and Setlow, 1979b), and it could be activated inside the isolated forespores when incubated with Mn^{2+} plus ionophore (X 537A) (Singh and Setlow, 1979b). Interestingly, *B. megaterium* accumulated not only Ca^{2+} but also Mn^{2+} during sporulation, therefore, one can ask how phosphoglycerate mutase and its substrate (3-PGA) coexisted inside the forespore without interaction. A possible explanation was provided by Singh and Setlow (1979b) who proposed that in the developing spore the level of free Mn^{2+} was low resulting in the occurrence of inactive enzyme. It was further postulated that a metal binder might be synthesised during the sporulation which is degraded upon spore germination. This suggestion was examined and results showed that a mutase inhibitor accumulated inside the developing forespore. This accumulation coincides with the accumulation of a stable pool of 3-PGA but well before DPA synthesis. This mutase inhibitor is present only in forespores and dormant spores and no appreciable activity was detected either in vegetative cells or in the mother cell compartment and a significant decrease (>95%) in activity was noticed in germinated spores. Further the activity was lost when pretreated with chymotrypsin (unpublished data).

This inhibitor was isolated and purified from dormant spores and was found to be a protein with a molecular weight of 11,000. It binds Mn^{2+} preferentially over Ca^{2+} with a binding constant of 1.3×10^5 M^{-1} for Mn^{2+} and 1.7×10^5 M^{-1} for Ca^{2+}. The association constant for Mn^{2+} was in the order of 47 µM/unit of mutase inhibitor as determined by Scatchard plots analysis (Scatchard 1949). This analysis indicated the existence of high and low affinity binding sites on the mutase inhibitor which suggested that the mutase inhibitor tied up the free Mn^{2+} ions in the forespores at least where DPA concentration was less than 10% of the maximum as shown in figure 1 (arrows 1 and 2), because once DPA starts accumulating in the forespores it binds not only Mn^{2+} but also other divalent metals as DPA (Singh, 1982) is a known metal chelator. Since DPA begins to accumulate 1.5 h after 3 PGA accumulation the possibility of DPA inactivating phosphoglycerate mutase could be ruled out (Singh and Setlow, 1978a). From these data, it is still not possible to predict how mutase inhibitor selectively binds free Mn^{2+} inspite of the fact that more than 95% of Mn^{2+} from medium is taken up by the sporulating cells. It has already been demonstrated (Singh and Setlow, 1979b) that about 70% of the total Mn^{2+} is bound to DPA which is released upon spore germination along with DPA. Therefore, it is likely that the remaining Mn^{2+} is bound to mutase inhibitor at least partly if not completely. This fraction of the Mn^{2+} seems to be critical for phosphoglycerate mutase activity in forespores at least where the spore core is very much hydrated and could still accumulate stable 3-PGA pool.

It is difficult to suggest that the mutase inhibitor completely inhibits phospho-glycerate mutase in the developing spores but it may contribute to its regulation although other factors like *in vivo* water content (Goud and Dring, 1975) and internal pH of forespores and spores (Setlow and Setlow, 1980) might also be

responsible in the inactivation of phosphoglycerate mutase activity. Further work is needed to locate the site of accumulation of the phosphoglycerate mutase inhibitor and to study the kinetics of inactivation of phosphoglycerate mutase by its inhibitor in the developing spores.

Acknowledgement

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Reactivity of fluorodinitrobenzene with intact human leucocytes: Examination of sites of action and the molecular entities involved in interaction

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Abstract. The epsilon amino groups of lysine and to a lesser extent amino groups on phosphotidyl ethanolamine and phosphotidyl serine situated on cell membranes of normal human leucocytes have been identified as the sites of dinitrophenylation. *In situ* conversion of 10,000 hydrophylic amino sites into hydrophobic sites on cell surfaces causes conformational changes in cell membranes with exposures of leukaemic cell-specific neo-antigens on tagged cell surfaces.

Keywords. Dinitrophenylated leucocytes; leukaemia-associated antigenicity; hydro-phylic-hydrophobic interconversions; dinitrophenylated-epsilon lysine; dinitrophenylated phosphatidyl ethanolamine; dinitrophenylated-phosphatidyl serine.

Introduction

Earlier studies from this laboratory (Sahasrabudhe 1968, 1980; Sahasrabudhe *et al.*, 1971a; Joshi *et al.*, 1981) have shown that normal human leucocytes when complexed with 1-fluoro-2,4, dinitrobenzene (FDNB) in proportions of 10^4 molecules of FDNB per cell, acquire *de novo* properties such as increased electronegativity on cell surfaces (Prema *et al.*, 1977), mobility of cell surface macromolecules (Sahasrabudhe, 1980), exposures of receptor sites for Concanavalin A (Con A) and wheat germ agglutinins (WGA) on cell surfaces (Madyastha *et al.*, 1975; Barth *et al.*, 1976), very similar to those exhibited by leukaemic cells. These tagged cells when inoculated into rabbits, horses and humans, elicited humoral and cell-mediated immune responses specific against human leukaemic cells (Sahasrabudhe *et al.*, 1971a, b, 1974; Sahasrabudhe, 1973). This induction of antigenicity in chemically modified normal human cells,

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exclusively specific against leukaemic cells, has hitherto been unknown in the past. That the immunological responses were not caused by hapten dinitrophenylated moieties (Karande and Sahasrabudhe, 1978) or by the possible dissimilarities in the HLA profiles of the innoculated human cells has amply been proved and demonstrated (Sahasrabudhe *et al.,* 1972a, b). Since dinitrophenylation of 10,000 sites on cell surface involves *in situ* conversion of 10,000 hydrophylic sites into hydrophobic sites, a conformational change dictated by thermodynamic considerations, takes place pushing the newly formed hydrophobic sites (10,000 of them) into deeper hydrophobic microenvironments of the cell membranes, out of contact from the surrounding aqueous medium. This upheaval causes exposure of new macromolecular entities (which were hidden in the cell membrane in cryptic forms) on cell surfaces. It is these newly exposed macromolecular entities which mimic the leukaemic cell surface characteristics (Sahasrabudhe, 1980).

The proportions of FDNB molecules per cell appears to play a critical role in the exposure of leukaemic cell mimetic characteristics in tagged cell surfaces. Optimum level of tagging for eliciting leukaemia-cell-mimetic properties was shown to be 10,000 FDNB molecules per cell. With a few thousand less molecules or a few thousand more molecules of FDNB per cell the leukaemia specific immunological response is either not elicited or partially elicited (Prema *et al.,* 1978).

In order to understand the mechanism involved and why FDNB molecules in the range of 10,000 molecules per cell are required for transforming normal human cells into leukaemic-cell-mimetic entities, it is necessary to known the sites of action of FDNB on cell membranes and what happens to those macromolecular moieties that get complexed with FDNB in the post *in situ* hydrophylic-hydrophobic interconversion phases. This has been attempted in this communication using [^{14}C]-labelled FDNB.

Materials and methods

Separation of human leucocytes and tagging with [^{14}C]-labelled 1-fluoro-2, 4,dinitrobenzene.

Leucocytes from 'O' group normal healthy human donors were obtained as described earlier (Sahasrabudhe *et al.,* 1972a). Radioactive 1-fluoro 2, 4,dinitrobenzene uniformly labelled with [^{14}C] (specific activity 17 mCi/m mols) hereafter referred as [^{14}C]-FDNB, was obtained from Radiochemical Centre, Amersham, England. It was dissolved and diluted with normal saline to give two solutions having concentrations of 10^{14} molecules of labelled FDNB per ml and 10^{16} molecules of labelled FDNB per ml. One ml of each of the above solutions was added to two 1 ml aliquots of leucocytes cell suspensions containing 10^{10} leucocytes per ml. These mixtures were gently shaken for 10 min at room temperature and the tagged cells separated by centrifugation at 500 *g* for 10 min. Supernatant solution was discarded and the FDNB-tagged cell pellets were washed three times with normal saline (each time with 50 ml) to remove unreacted FDNB along with any FDNB that might have combined with loosely bound proteins on cell surfaces (Gharpure, 1977). The washed cell pellets were

resuspended in sucrose, Tris, magnesium chloride buffer (pH 7.2) containing sucrose 0.25 M, Tris 0.01 M and magnesium chloride 0.005 M (Warren and Glick, 1967) and homogenized. The homogenate was centrifuged at 500 g for 10 min for the separation of nuclear material. The nuclei-free supernatant was centrifuged at 7500 g for 10 min in a Sorval RC-50 centrifuge to separate mitochondrial preparations. The supernatant after removal of mitochondrial pellet was centrifuged for 2 h at 105,000 g in a Spinco 65 B ultracentrifuge. The pellet obtained was further fractionated by a second ultracentrifugation in a sucrose gradient as described by Warren and Glick (1967) to obtain the membrane fraction.

Radioactivity in each of the above mentioned fractions was determined by counting aliquots in Beckman LS 100 Liquid Scintillation spectrometer.

Analysis of membrane proteins

Labelled dinitrophenylated (DNP-) membrane proteins were precipitated from the membrane fraction by the addition of 10% cold trichloroacetic acid. Cold (*i.e.* non-radioactive) DNP-membrane proteins were isolated from leucocytes tagged with non-radioactive FDNB using identical procedure with that used in the isolation of labelled DNP-membrane proteins and added as carriers to the latter. The mixtures were hydrolysed with 6 N HCl as described by Lederer and Lederer (1955). Fifty μg aliquots of membrane protein hydrolysates dissolved in 0.1 ml of methyl ethylketone-saturated water were chromatographed on Kiselghur column (Perrone, 1951). The column was gradually eluted with decreasing concentrations of methyl ethyl ketone in chloroform (75%, 45%, 30% and 0%), methanol and water. Absorbance at 360 nm of all the eluate fractions was measured in a Beckman Spectrometer model 26. Fractions showing absorbance at 360 nm greater than 0.05 were concentrated and their radioactivity determined. Individual DNP-amino acids, obtained either from protein hydrolysates or those eluted from various fractions collected after column chromatography, were further identified by chromatography on Whatman No. 3 filter paper with authentic samples of DNP-amino acids in at least two solvent systems (Smith, 1960). DNP-amino acids are easily recognised on filter paper chromatograms by their yellow colour. Epsilon DNP-lysine and DNP-arginine have Rf values in the same range. To distinguish one from the other, ninhydrin spraying was used. Lysine gives brown colour with ninhydrin.

Analysis of membrane lipids

Aliquots of dinitrophenylated cells were centrifuged at 500 g for 10 min and the pellet thus obtained was resuspended in normal saline. To this, 20 volumes of chloroform:methanol (1:1) mixture was added and the mixture shaken vigorously to extract the lipids. The chloroform-methanol layer containing the lipids was seperated and the solvent evaporated to dryness. The lipid residue was taken in minimum quantity of methanol and chromatographed on silica gel using chloroform:methanol:water (150:50:7 v/v) solvent system. DNP-phospholipids were identified by yellow colour while the unreacted amino-lipids were identified by staining with ninhydrin. The yellow spots containing DNP-phospholipids were scraped off and dissolved in methanol. These were scanned at

345 nm for absorption which is specific for DNP-phosphatidyl serine and DNP-phosphatidyl ethanolamine (Harris *et al.,* 1954). These were further identified by their Rf values and UV absorption spectra. To determine the radioactivity in individual spots, the material was taken in 10 ml Bray's scintillation fluid (naphthalene 18g, 2,5,diphenyloxazole (PPO) 1.2 g 2,2'-*p*-Phenylene-bis-(5-phenyloxazole) (POPOP) 0.05 g dissolved in 1-4 dioxan 264 ml, methanol 30 ml and ethylene glycol 6 ml) and radioactivity counted.

Results and·discussion

When ratio of molecules of FDNB per cell was 10,000, only 46% of the added radioactivity was incorporated in cellular components. When the· proportion of FDNB molecules added was increased to 10^6 per cell, the fraction incorporated into cell components decreased to 31%. The remaining 54% and 69% radioactivity respectively, were lost either as unreacted FDNB or in the form of complex with loosely bound surface proteins which leached out during washing (Gharpure, 1977). Table 1 shows that major portions of radioactivity was present in the membrane fraction when tagged cells bearing 10^4 molecules of FDNB per cell were prepared. Mitochondrial, nuclear and cytoplasmic fractions also incorporated low amounts of radioactivity and could be an artifact of the method.

Table 1. Distribution of radioactivity in the subcellular fractions of leucocytes tagged with [^{14}C]-FDNB.

	10^4 molecules of [^{14}C]-FDNB per cell		10^6 molecules of [^{14}C]-FDNB per cell	
	cpm		cpm	
Whole cells	8400	(46%)[a]	495,562	(31%)[a]
Membrane fraction	5400		7,200	(1.4%)[b]
Mitochondrial	1200	(14%)[b]	211,700	(43%)[b]
Nuclear	1100	(13%)[b]	154,200	(31%)[b]
Cytoplasmic	700	(8%)[b]	122,462	(25%)

Total radioactivity used in the case of 10^4 and 10^6 molecules of FDNB were 18,000 and 1,586,475 cpm respectively. Out of the total radioactivity, 54 and 69% was lost as unreacted FDNB or that complexed with loosely bound proteins in the washings.

[a] % of total radioactivity used.

[b] % of radioactivity incorporated into whole cells.

In the preparation of tagged cells, a calculated amount of FDNB was added to a fixed number of cells expecting that all the cells would have the same number of FDNB molecules per cell. In actual practice, it is likely that while majority of cells would correspond to the desired proportion of FDNB molecules per cell, some cells may have acquired more or some less number of FDNB molecules per cell. The cell membranes were postulated to contain 10^6 amino groups on cell surfaces

(Mehrishi, 1970... If all the cells had been tagged at 10^4 FDNB molecules per cell, then the chances of FDNB reacting with mitochondrial or nuclear or to cytoplasmic fractions would be nil. The observation that these fractions contain some radioactivity even when the proportion of 10^4 molecules of FDNB per cell was used, suggests that some cells may have reacted with more than the predicted number of FDNB molecules per cell thus enabling FDNB molecules to enter the cytoplasm etc. When the proportion of FDNB molecules added per cell was increased one hundred-fold *i.e.* to 10^6 FDNB molecules per cell, only marginal increase in radioactivity in membrane fraction was seen, whereas the radioactivity in mitochondrial, nuclear and cytoplasmic fractions increased by 150 to 200 fold (table 1).

Distribution of radioactivity in various DNP-amino acids and DNP-phospholipids is shown in figures 1 and 2 and table 2. Figure 1 shows the elution pattern of DNP-amino acids (absorbance at 360 nm) in the hydrolysates of proteins obtained from membrane fractions. Figure 1 shows one major and two
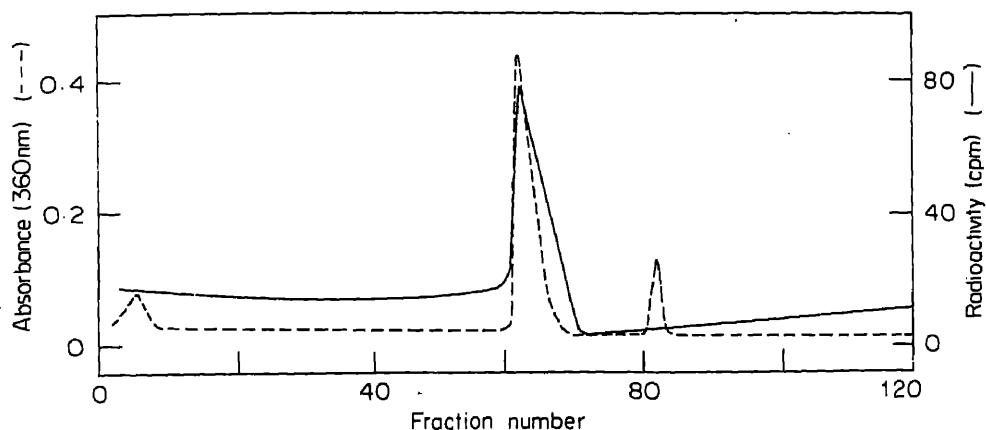
Figure 1. Chromatography of the hydrolysate of FDNB-treated membrane proteins. Membrane protein hydrolysates from dinitrophenylated human leuocytes were chromatographed on Kiesulghur column and absorption at 360 nm (for DNP-amino acids, dotted line in the figure) and radioactivity (solid line in the figure) determined in the eluted fractions.

minor 360 nm absorption peaks corresponding to DNP-amino acids. The major peak which also showed radioactivity was shown to be due to dinitrophenylated epsilon lysine. This fact was confirmed by running the samples along with authentic DNP-amino acid samples on paper chromatography and their reaction with ninhydrin (figure 2). The remaining two minor peaks exhibiting absorption at 360 nm and showing insignificant radioactivity, could not be identified as they were present in trace amounts.

DNP-phospholipids obtained from the membrane fractions were separated by thin layer chromatography and the amounts of DNP-phosphatidyl serine and DNP-phosphatidyl serine and DNP-phosphatidyl ethanolamine were estimated by their absorption at 345 nm. The amounts of the two DNP-phospholipids and their respective radioactivities are given in table 2.
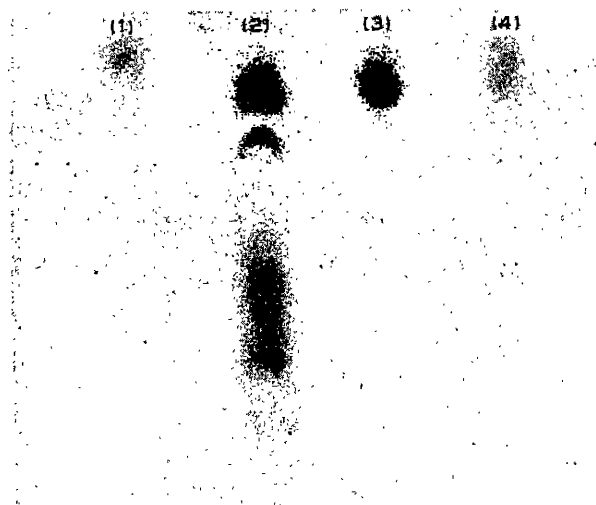
Figure 2. Paper chromatography of membrane protein hydrolysates from dinitrophenylated human leucocytes. 1. "Major peak" eluate from kiesulghur column chromatography (see figure 1), 2. Total membrane protein hydrolysate, 3. ε-Amino dinitrophenylated lysine, 4. Dinitrophenylated arginine.

Table 2. Dinitrophenylation of membrane phospholipids in leucocytes tagged with [^{14}C]-FDNB.

	10^4 molecules of [^{14}C]-FDNB per cell		10^6 molecules of [^{14}C]-FDNB per cell	
	Absorbance (345 nm)	Radioactivity (CPM)	Absorbance (345 nm)	Radioactivity (CPM)
DNP-Phosphatidyl ethanolamine	0.357	180±12	0.469	217±29
DNP-Phosphatidyl serine	0.174	106±10	0.277	96±9

These findings indicate that with 10,000 molecules of FDNB per cell (*i.e.* the proportion which exposes *de novo* leukaemic cell mimetic properties on cell surfaces) the amount is just sufficient to dinitrophenylate only the membrane constituents. With larger amounts, more FDNB is available for entry into the cell for dinitrophenylation of mitochondria, nuclei and cytoplasmic constituents. But this adversely affects the capacity of tagged cells to exhibit its leukaemia antigenicity (Prema *et al.*, 1978). Apparently epsilon amino group of lysine and to a lesser extent phosphatidyl ethanolamine and phosphatidyl serine are the only constituents in the membrane which were dinitrophenylated. Dinitrophenylation of phospholipids, as has been stated earlier, is considered to be an artifact arising from cells which have reacted when a higher amount of FDNB molecules per cell than the desired 10,000 molecules was used. The exact mechanism of exposure of leukaemia specific neo-antigens on tagged cells and the role dinitrophenylation of epsilon lysine plays in these transformations is not clear. That the DNP-hapten has no role in creation of leukaemia antigenicities has been shown by the fact that tagged cells do not respond to specific anti-DNP antibodies (Karande and Sahasrabudhe, 1978). By dinitrophenylation, we are converting *in situ* 10,000 hydrophylic groups on cell surfaces into an equal number of hydrophobic groups (Iyer *et al.*, 1979, Haseley and Biltonen, 1975). The presence of 10,000 hydrophobic groups on cell surface in contact with aqueous environment would require expenditure of considerable energy, without which there would be a natural tendency for the newly formed hydrophobic groups to sink deeper into the inner hydrophobic microenvironment (Singer, 1971). This *in situ* conversion of a large number of hydrophilic sites into hydrophobic site would cause conformational changes and possibly convulsions in cell surface topography causing exposure of neoantigens on tagged cell surfaces which mimic leukaemia-associated antigenicity.

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[^{31}P]-Nuclear magnetic resonance spin lattice relaxation in lecithin reverse micelles

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Abstract. [^{31}P]-Nuclear magnetic resonance (NMR) spin lattice relaxation times (T_1) have been measured for lecithin-nonpolar solvent-water as a function of added water for three solvents, namely, benzene, carbon tetrachloride and cyclohexane. In benzene and carbon tetrachloride systems, where spherical reverse micelles are formed, [^{31}P]-NMR T_1 values increase linearly with added water. However, in cyclohexane, the trends in the [^{31}P]-T_1 values indicate very different micellisation processes. Even at the lowest concentration of added water, the [^{31}P]-T_1 values in this solvent are substantially larger than the corresponding values in benzene and carbon tetrachloride, which is attributed to the intramolecular chlorine-phosphate interaction being the weakest in cyclohexane. At a higher water content of six mols of water per mol of lecithin in cyclohexane solvent, the [^{31}P]-T_1 values show a sharp decrease indicating a sudden change in the dynamics of the phosphate group, and this confirms the on set of 'reverse micelle-to-liquid crystalline' phase transition observed in this system by other spectroscopic and physical techniques.

Keywords. [^{31}P]-NMR; spin lattice relaxation; lecithin reverse micelles.

Introduction

There has been a rapid growth of interest in recent years in the study of phospholipid aggregates in nonpolar media since these aggregated structures often 'model' faithfully the nature of cell membranes. Such aggregation leads to reverse micelles with a polar interior and a nonpolar outer surface. These aggregates can solubilize water in their interior as small water 'pools'. Several proton magnetic resonance studies on such reverse micellar systems are available, in which the phospholipid-water interactions have been studied (Davenport and Fisher, 1975; Henrikson, 1970; Shaw *et al.*, 1973; Walter and Hayes, 1971; Wells, 1974).

Two reports in the literature contains a specific reference to [^{31}P]-spin lattice relaxation in lecithin reverse micelles. Klose and Stelzner (1974) have investigated the system lecithin-benzene-water, and the dependence of their [^{31}P]-T_1 values on

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Abbreviations used: NMR, Nuclear magnetic resonance TLC, thin layer chromatography.

added water show a roughly sigmoidal (i.e., S-shaped) dependence, with unexplained plateau regions at either end of the water concentration scale which these authors refer to as 'breaks', and a sharp increase in between. On the other hand, by performing a similar [^{31}P]-NMR relaxation experiment on lecithin-carbon tetrachloride-water system, Fung and McAdams (1976) have observed that the [^{31}P]-T$_1$'s increased linearly with added water, without any 'break'. Clearly, it would be desirable to have a further understanding of the lecithin [^{31}P]-relaxation in these systems.

From our earlier physical (Kumar, *et al.*, unpublished observations) and spectroscopic (Kumar and Raghunathan, 1982) studies there is strong empirical evidence to show that in lecithin-cyclohexane-water system at a water concentration of six mols per mol of lecithin, the isotropic reverse micellar system changes to an anisotropic liquid crystalline state. Indeed, water diffusion in the above system, studied by the magnetic field gradient-spin echo method (Kumar and Raghunathan, unpublished observations), is reduced by as much as 30% compared with that of normal, bulk water. In this communication, we not only report a re-examination of the [^{31}P]-spin lattice relaxation of lecithin in benzene and carbon tetrachloride as a function of the solubilised water, but also present the first observation of [^{31}P]-NMR spin lattice relaxation times of the system lecithin-cyclohexane-water as a function of added water. As will become apparent, the cyclohexane system is a very interesting one in its own right.

Materials and methods

All the solvents used were of Analar grade, which were further dried and distilled. Chromatographically homogeneous egg lecithin was extracted by the method of Singleton *et al.* (1965). Thin layer chromatography (TLC) on silica gel G (Merck) was used for checking the purity of the extracted lipid. The phosphatide extractions from egg yolk in a 9:1 chloroform-methanol mixture were first applied to the TLC plates and then developed by solvent system chloroform-methanol-water (65:25:4, v/v) in a solvent-vapour saturated chamber. Iodine vapour was used for identifying the chromatographed material. A single spot on the TLC plate confirmed the purity of the lipid. After evaporating away the solvent mixture, the residual solid lipid was dried under vacuum (~50 microns) for about 6 h and then dissolved in the required dry solvent. These solutions, as well as the triple-distilled water used in this work, were deoxygenated by purging with a current of dry nitrogen gas before the NMR measurements.

[^{31}P]-NMR results were recorded on a Varian XL-100 FT NMR spectrometer at a [^{31}P]-Larmor frequency of 40.5 MHz. phosphoric acid 85% in a capillary tube was used as a reference, with a C$_6$D$_6$ external 'lock' for field-frequency control. The relaxation times (T$_1$), determined by the standard 180°-T-90° pulse sequence, were accurate to within ±5%.

All measurements were carried out at 25 ± 1°C over a range of R values, where R is defined as the molar concentration ratio (= [H$_2$O]/[lecithin]).

Results and discussion

Figure 1 shows the variation of [³¹P]-spin lattice relaxation time of the choline headgroup with increasing water concentrationtion for the three systems, namely, lecithin-benzene-water, lecithin-carbon tetrachloride-water and lecithin-cyclo-hexane-water. Even at first glance, the point which is striking here is that
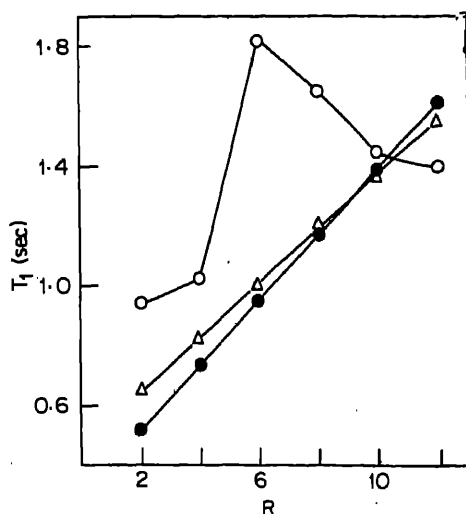
Figure 1. Variation in [³¹P]-NMR T_1 values of lecithin head group in lecithin-benzene, lecithin-carbon tetrachloride and lecithin-cyclohexane systems as functions of added water.

Benzene system, (Δ); carbon-tetrachloride system, (●); and cyclohexane system, (○).

though all the three solvents are highly nonpolar (having almost the same dielectric constant of around 3), the cyclohexane system behaves entirely differently from the other two. This leads to the inference that micelles formation takes place in different ways in different solvents, and it is perhaps useful to make the cautionary remark here that one ought not to extrapolate the apparent physical state of the micelle from one solvent to another.

From figure 1 it is clear that [³¹P]-T_1 in benzene and carbon tetrachloride systems increase linearly with added water. In the water concentration range studied, the slopes of our T_1 curves for these solvents are in quantitative agreement with each other and with that found by Fung and McAdams (1976) in carbon tetrachloride (the only solvent these authors used). Like Fung and McAdams (1976), we do not also find the distinct 'breaks' reported by Klose and Stelzner (1974). It should also be noted here that in a subsequent study, Klose *et al.* (1978) have reported that the lecithin-benzene system shows considerable temperature-hysteresis and 'ageing' effects and that small amounts of water change the properties of their system drastically; in view of their findings, these authors have stated that the earlier conclusions of Klose and Stelzner (1974) are uncertain.

In the extreme-narrowing limit an increase in [³¹P]-relaxation time (T_1) means a decrease in the rotational correlation time of the phosphate group, i.e., an increase

in its mobility. At very low concentrations of water, the positively charged trimethylammonium group and the negatively charged phosphate group would interact with each other strongly, with consequent severe hindrance in the internal rotation of the phosphate group. Here we stress that, as distinct from the intermolecular interaction between choline and phosphate groups from adjacent lipid molecules in the case of bilayer vesicle structures in aqueous medium (Buldt and Wohlegemuth, 1981), the choline-phosphate interaction usually envisaged in reverse micellar systems is intramolecular, being brought about by a water molecule becoming hydrogen-bonded to the phospholipid head group (see below).

Extensive proton NMR chemical shift and T_1 results for water added to lecithin in the three nonpolar solvents perdeuterobenzene, carbon tetrachloride and perdeuterocyclo-hexane (Kumar, 1982; Kumar and Raghunathan, unpublished observations) have established that, in the former two solvent media, one H_2O is tightly bound per polar headgroup at all water concentrations whereas it is much more loosely bound in the cyclohexane medium. The conclusion that one molecule of water becomes bound to the headgroup is in excellent agreement with the deuterium NMR T_1 results of Fung and McAdams (1976) who have, however, worked only with the lecithin-D_2O-carbon tetrachloride system. The proposed binding of water to the zwitterionic headgroup is represented in figure 2. This 'folded' arrangement derives support from the quantum mechanical studies of Pullman and Berthod (1974) on the energetically preferred conformations of the polar heads of phospholipids.
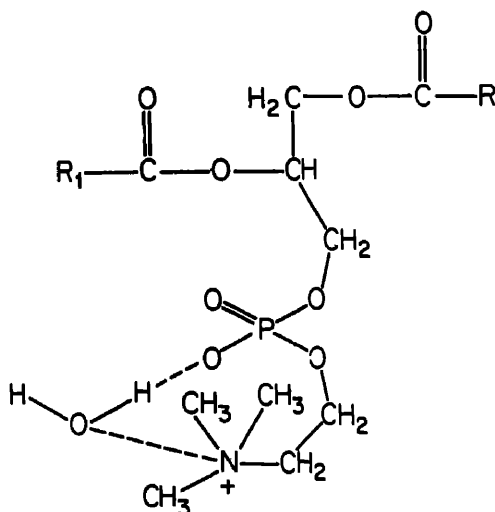
Figure 2. Model of interaction of water with the polar headgroup of lecithin reverse micelles.

With increasing R, this choline-phosphate interaction weakens progressively due to the exchange of the bound water with water in the central 'pool', thereby leading to greater relaxation of the headgroup. The water-binding has been

observed to be the weakest in cyclohexane in the proton NMR studies of Kumar and Raghunathan (unpublished observations), and this explains the observed difference in the $[^{31}P]$-T_1 values in figure 1 at initial R values (e.g., $R = 2$) between the cyclohexane system on the one hand ($T_1 = 0.90 \pm 0.03$ s) and the benzene and carbon tetrachloride systems on the other (T_1 about 0.60 s). Further spectroscopic studies on the same systems in our laboratory using 'polarity' probes (Kumar and Raghunathan, 1982) have not only confirmed the exchanged between the bound and pool water at high R values in the above-mentioned solvents, but also the weak binding of the H_2O to the headgroup in cyclohexane solvent.

As already noted, the other remarkable feature of figure 1 is that phosphorus T_1 values in cyclohexane increase upto the T_1's upto $R = 6$ shows that the mobility of the phosphate group increases upto this point. At further concentrations of added water, a sharp decrease in the mobility of the phosphate group is heralded, suggesting that some molecular rearrangements involing the headgroup are taking place in cyclohexane medium at an R value of 6.

A possible mechanism that could be invoked to explain the above $[^{31}P]$-NMR T_1 data is as follows: the change in T_1 values at $R = 6$ can be rationalised as being due to the onset of an ordered liquid-crystalline phase, complemented by the inter-calation of cyclohexane molecules between successive hydrocarbon chains of the lecithin $-(CH_2)_n-$ 'tail' in the hydrophobic region.

The results of our other spectroscopic investigations pertinent to this point are the following: our studies using 'polarity' probes such as the NO_3^- ion and anilinonaphthalene sulphonic acid (Kumar, 1982; Kumar and Raghunathan, 1982) show that (a) the amount of water present in the organic solvent phase is negligible and (b) in the cyclohexane system in particular, intercalation of the organic solvent could be occurring gradually even at lower R values, eventually 'triggering' the phase transition around $R = 6$. If our proposed cyclohexane 'intercalation' mechanism is correct, then the protons of the lipid tail should be bathed in this organic solvent and therefore, not be in contact with the added water. Indeed, proton T_1 values of the '$(CH_2)_n-$ tail in (perdeuterated) cyclohexane remain constant at 0.45 seconds for R values between 2 and 10 (Kumar and Raghunathan, unpublished observations), lending support to our proposal.

Our present study independently confirms the occurrence of the reverse micelle-to-liquid crystalline phase transition indicated by electron microscopy and a variety of other physical and spectroscopic measurements made in our laboratory (Kumar and Raghunathan, 1982).

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Relationship between physiological energy expenditure and biomechanical patterns associated with erect standing

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Abstract. The deviation of the static weight-bearing patterns under the feet of a lower extremity handicapped person may be measured quantitatively in terms of the Static Weight-Bearing Index. This paper describes the correlation between this index and the physiological energy expenditure associated with the erect standing posture. The Static Weight-Bearing Index can be conveniently used for evaluation of the functional status of the human lower extremity system in stance in case of lower extremity disabled persons who maintain basic weight-bearing mode involving both the limbs in double support condition.

Keywords. Static weight-bearing; performance evaluation; disability; energy expenditure; biomechanical patterns.

Introduction

Many studies on weight-bearing patterns of human feet have been aimed at assessing the success of treatment and effectiveness of rehabilitation in lower extremity disabled people as well as for exploring the fundamental biomechanical characteristics of the human lower extremity system. The studies on human locomotion have been concerned with the dynamic weight-bearing under moving feet (Fenn, 1930; Elftman, 1934; Schwartz and Heath, 1947, 1949; Saunders *et al.*, 1953; Stokes *et al.*, 1974; Arcan and Brull, 1976; Miyazaki and Iwakura, 1976). The static weight-bearing patterns have been investigated upon using different techniques to correlate the variations in such patterns with the stance disability of these patients (Shambes and Waterland, 1970; Chodera and Sharma, 1977; Chodera and Cterceteko, 1979; Staros, 1965; Ghosh *et al.*, 1979; Tibarewala and Ganguli, 1982). Using a system of strain gauge load cells, a biomechanical evaluation of human lower extremity disability leading to defining of a Static Weight-Bearing Index as a quantitative measure of this disability during erect standing (to be published elsewhere) was carried out. To evaluate the usefulness of this parameter it was necessary to correlate it with other established measures of human performance.

Abbreviations used: NG, normal group; LEH, lower extremity handicapped; PPC, post-polio cases; BKP, below-knee amputes using patellar-tendon-bearing prostheses; ACU, axillary crutch users; SWB, static weight-bearing; E, standing energy; BW, percentage body weight.

Measure of physiological energy expenditure by indirect calorimetery (Consolazio *et al.*, 1963; Durnin and Passmore, 1967) is considered as good as directed measurements. As performance is related to energy expenditure, an attempt was made to establish the relationship between the standing energy expenditure and Static Weight Bearing Index before recommending the latter for routine clinical applications.

Materials and methods

The test samples comprised of a normal group (NG) composed of 6 healthy, able-bodied, adult males with sedantry habits and a group of 23 lower extremity handicapped (LEH) consisting of 8 post-polio cases (PPC) affected on one side only, 6 unilateral below-knee amputees (traumatic) using patellar-tendon-bearing prostheses (BKP), and 9 auxillary crutch users (ACU). The personal data of these subject groups have been presented in table 1.

Table 1. Personal data of subjects (mean ±S.D.).

Group (number)	Age (yrs)	Height (m)	Body-weight (kg)	Weight of appliance (kg)
NG (6)	26.5±3.02	1.66±0.07	47.07±7.73	—
PPC (8)	21.1±3.4	1.55±0.05	39.73±5.96	—
BKP (6)	28.5±6.7	1.67±0.06	49.18±3.95	2.48±0.26
ACU (9)	25.11±3.9	1.62±0.07	48.01±7.09	2.53±0.26

Static weight-bearing (SWB) patterns under the feet of each subject were determined according to Tibarewala and Ganguli, (1982) and the SWB index was computed for each pattern according to Tibarewala and Ganguli (to be published else where). While a set of two indices (one for each foot) was determined for the subjects belonging to NG, BKP and PPC, only one SWB index (for healthy foot) along with the percentage of body weight transmitted through each crutch was determined for the ACU group. It may be noted that the method of measuring SWB patterns divides a foot into six parafrontal zones and measures the corresponding patterns as a set of six ordered numbers, each number representing the percentage body weight being transmitted through a particular zone. Further, SWB index for a particular pattern is the root-mean-square deviation of the set of numbers representing the mean normal pattern (Tibarewala and Ganguli, 1982).

The standing energy expenditure (E) was determined by indirect calorimetery (Consolazio *et al.*, 1963) and multiple regression analysis were conducted with E as the dependent variable. Since the list of independent variables for the NG, PPC, and BKP was different from that of ACU, two separate regression analyses were attempted: one for the ACU alone, and another for the combined group consisting of NG, BKP and PPC.

Results and discussion

In case of auxillary crutch users, the scatter diagram representing the variation of standing energy expenditure E with the SWB index (figure 1A) indicated the presence of a linear trend. In terms of the correlation coefficient, a negatively linear regression with r = −0.5 might have been obtained, without considering any other factor. Inclusion of the percentage body weight (BW) transmitted through each crutch as additional independent variables led to a multiple regression equation (equation 1) with a value 0.68 for the multiple correlation coefficient. The estimated value of E from this equation have been plotted against the observed values in figure 1B.

$$E = 171.72 - 4.72 \text{ SWBI} - 8.85 \text{ (\% BW under the right crutch)}$$
$$+ 7.30 \text{ (\% BW under the left crutch)} \qquad (1)$$

$$(R = 0.68)$$
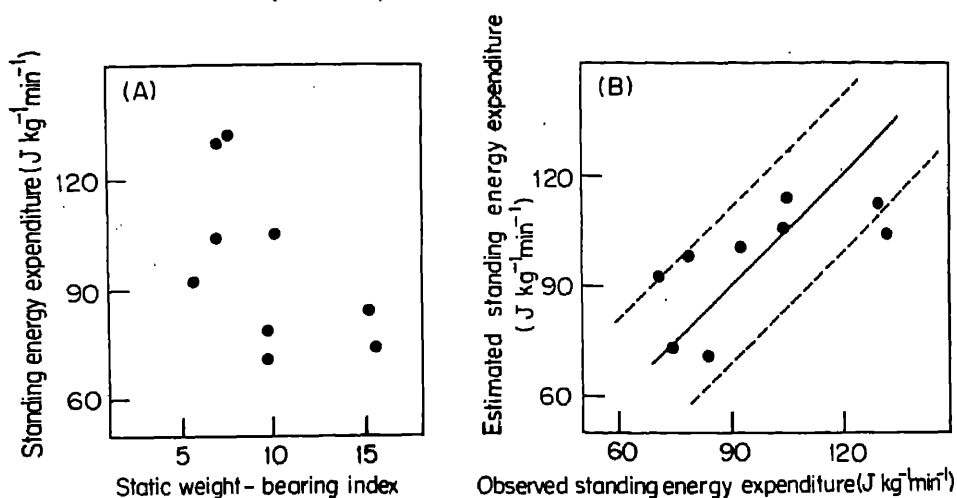
Figure 1. A. Standing energy expenditure *versus* static weight-bearing index (for auxillary crutch users). **B.** Standing energy expenditure estimated from equation 1 *versus* observed standing energy expenditure (for auxillary crutch users).

For the rest of the test subjects including NG, PPC and BKP, the standing energy expenditure has been plotted against the SWB indices in figure 2A. Since the values of the two SWB indices for the normals were nearly equal, an arbitrary choice was made to consider the left side of the normals with the healthy side of the LEH, and to specify the corresponding SWB index as SWBI (HS/L). Similarly, the right side of the normals was considered with the affected side of the LEH and the corresponding SWB index has been specified as SWBI (AS/R). Existence of linear correlation between the standing energy expenditure and the two SWB indices was apparent from this scatter diagram. This observation was further supported by computations whereby correlation coefficients between SWBI (HS/L) and E, and
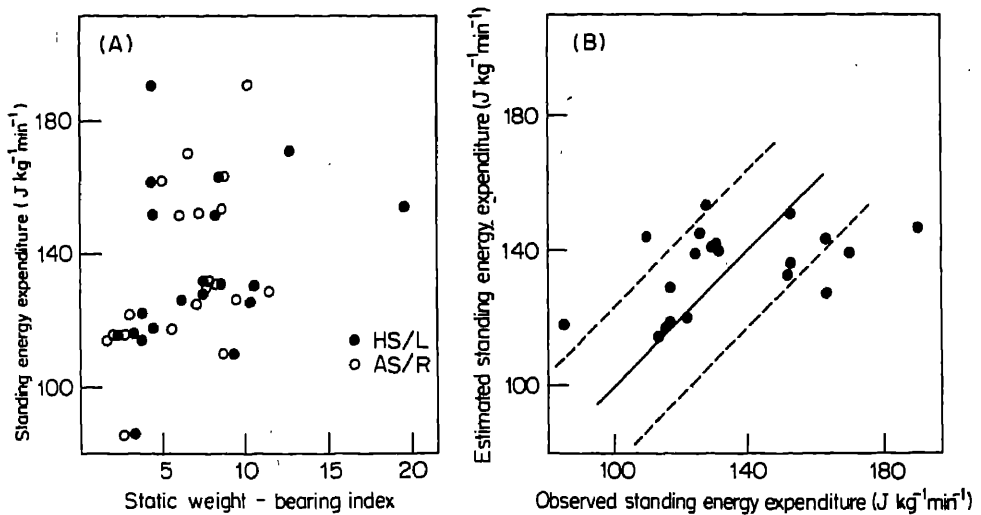
Figure 2. A. Standing energy expenditure *versus* static weight-bearing indices (for normals, below-knee amputees and post-polio cases). **B.** Standing energy expenditure estimated from equation 2 *versus* observed standing energy expenditure (for normals, below-knee amputees and post-polio rehabilitees).

between E and SWBI (AS/R) were found to be 0.30 and 0.45 respectively. Multiple regression of E on the two SWB indices simultaneously improved the correlation coefficient upto 0.483. The partial correlation coefficients were found to be 0.114 between E and SWBI (HS/L), and 0.365 between E and SWBI (AS/R). This indicated the larger influence of SWBI (AS) compared to that of SWBI (HS) on the standing energy expenditure. The resulting multiple regression equation has been represented as equation 2.

$$E = 106.372 + 0.653 \text{ SWBI (HS/L)} + 3.677 \text{ SWBI (AS/R)} \qquad (2)$$

The values of E estimated from this equation have been plotted against the observed values in figure 2B. As before, the central line and the dotted lines represent the 'zero error' estimates and the 'standard error' zone respectively.

The regression equation 1 has a negative coefficient of the SWBI which means that the standing energy expenditure of axillary crutch users decreased with an increase of the SWB index. This derivation is paradoxial in the sense that it demands a high energy expenditure with near-normal SWB patterns. This paradox, however, can be explained easily by considering the three point weight-bearing of the ACU which is very much different from the normal weight-bearing. The normal SWB pattern which is optimum for the two point stance, may not be so in the other case as a result of which any attempt of an ACU to adopt the optimum SWB characteristics with a three-point weight-bearing might have caused an increase of the deviation from the normal SWB pattern but a decrease in the standing energy expenditure rate. In case of the normals and the lower extremity handicapped other than ACU, however, the coefficients of both the SWB indices

(in regression eg. 2) are positive indicating thereby that the standing energy expenditure increased with any increase in the deviation of SWB pattern from the mean normal pattern.

The regression analyses were repeated with different groups as separate strata whereby correlation coefficients between 0.3 and 0.8 were observed for each stratum, thus, eliminating the possibility of the observed correlation for the combined sample being spurious. Under such a situation, where the relationships having some amount of curvilinearity are approximated by linear regressions, it is safe to use the combined regressions rather than those obtained separately for each stratum (Cochran, 1977).

The regression equations obtained as a result of this investigation represent the relationships between the physiological energy expenditure incurred by the human body during erect standing — the most common static activity involving human lower extremity system, and the biomechanical measures of stance disability. Since the former have been recognised as reliable performance measures for long, the latter i.e. the biomechanical characteristics measured in terms of the SWB indices (and the portion of body weight transmitted through the crutches, in case of ACU) can now be used for performance evaluation of human lower extremity system in stance. On a comparative scale, an improved functional status in stance should be accompanied by lower values of the SWB indices. Because of entirely different three-point weight-bearing mode of standing, the functional status of auxiliary crutch users during the erect standing should not, however, be assessed on the basis of SWB index.

Acknowledgements

Appendix

A brief description of the system used for determining the SWB patterns and the measuring procedure involved is represented here to provide an insight to the readers without elaborate reference:

Description of the system

The instrumentation of the system comprised three units, namely, the transducers, the interface and, the indicator as shown in figure 3A.

Six strain gauge load cells were used as the transducers. Each load cell was composed of four pressure dependent resistances connected in a Wheatstone bridge fashion. When supplied with a constant voltage across two of its terminals, an imbalance voltage proportional to the load applied to the elements of this circuit is provided at the other two terminals. These load cells were fixed on a wooden platform and each had a thin aluminium strip covered with rubber padding at the

(A)
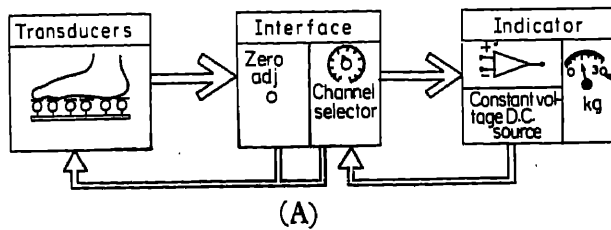
Figure 3. **A.** Flow diagram of the system for static weight-bearing studies. **B.** Placing the foot on load cell system.

top. The rectangular area thus formed by six parallel strips, constituted the support for the foot being investigated, while the other foot was supported by a dummy platform of matching area and height.

The interface served a dual purpose. Firstly, it contained a multi-pole selector switch, facilitating the activation of any one of the load cells at the investigators choice, thus making it possible to work with a single indicator. Secondly, it incorporated as its integrated portion, the arrangement for initial balancing (i.e. obtaining 'zero' output at 'zero' load on the load cells) of the Wheatstone bridge circuit. The indicator also had a dual role of a constant voltage source for the Wheatstone bridge supply and an amplifier-cum-display unit to indicate the imbalance voltage. The dial of the indicator was graduated directly in terms of kg.

Measuring procedure

Each load cell, with its corresponding part of the interface, was termed as one channel. Thus there were six numbered channels. The dummy platform and the load cell system were placed side by side so that the subject could stand with one foot on each, with toes placed towards channel 1 and heel towards channel 6 as shown in figure 3B. The selector switch was turned from 1 to 6 and the

corresponding load displayed on the indicator dial was noted. The process was repeated thrice and the average load on each channel determined. Following this, the position of the dummy platform and the load cell system was interchanged and the whole procedure repeated to obtain the average distribution under the other foot. Finally, these weight distribution figures were normalized by the subject's body weight so that the SWB pattern under a foot may be expressed as an ordered set of six numbers, each number representing the percentage body weight being transmitted to ground through the relevant portion of the foot.

Static weight-bearing index

Any static weight-bearing pattern (or SWB pattern), being a set of six ordered numbers, may be represented by an unique point in a six dimensional hyper space with rectilinear orthogonal axes so that the distances from origin along the axes represent the corresponding 'number'. This was the starting point of the approach. The points representing SWB patterns belonging to different types of feet formed separate clusters in different parts of the hyper space (to be published else where). Following this observation, it was felt that the difference between any two SWB patterns may be measured in terms of the distance between corresponding points in this space. In other words, the functional abnormality in erect standing may be measured by the departure of the corresponding SWB patterns from the mean normal pattern. Since the distance between two points in any space is a constant multiple of the root-mean-square (i.e. RMS), difference between the orthogonal rectilinear coordinates defining the points, a static weight-bearing index has been defined to measure the departure of any SWB pattern from the mean NG pattern as the RMS difference between the 'numbers' representing the two involved patterns.

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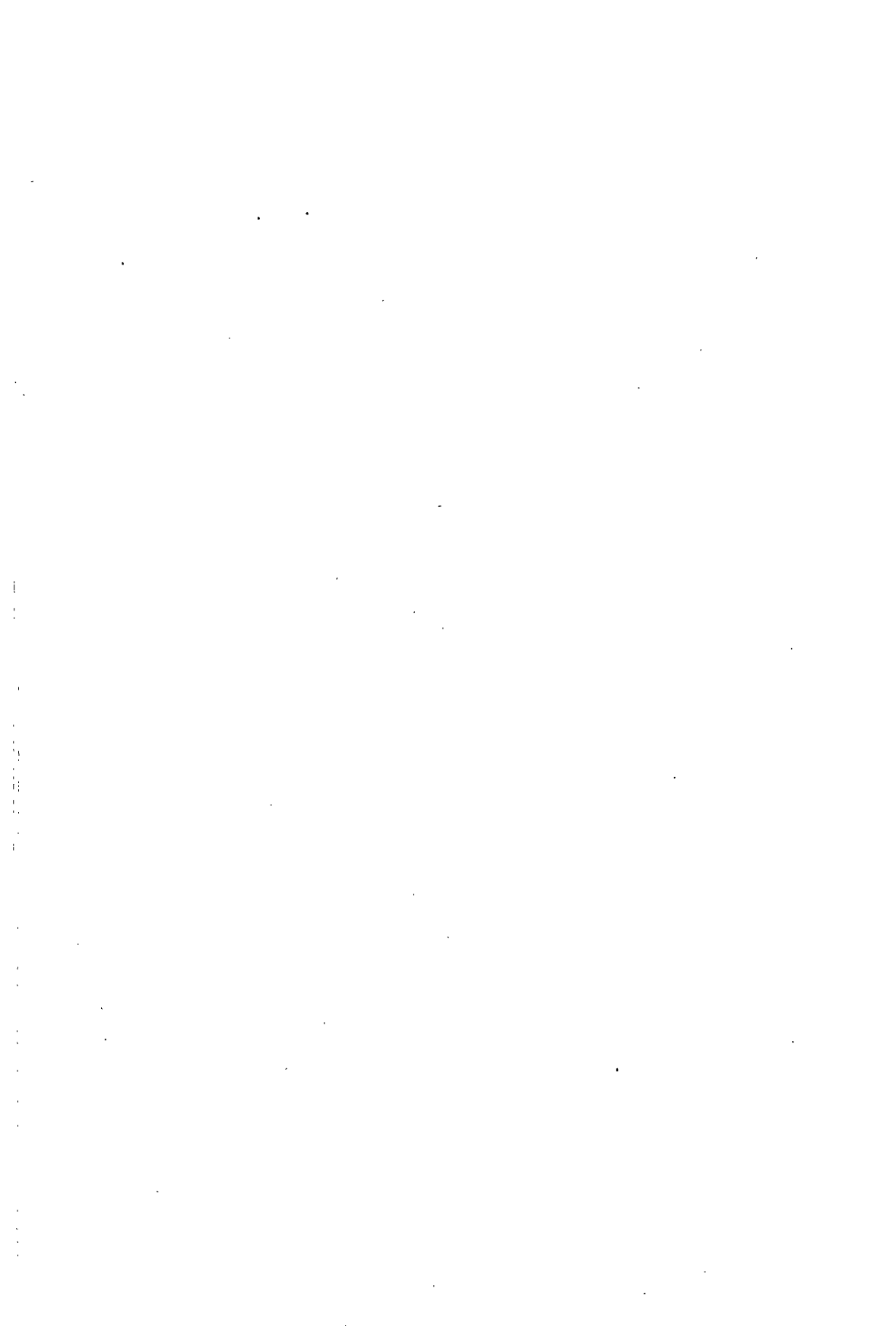
Effect of postweaning protein deficiency on the content and lipid composition of gray and white matter in neonatally undernourished rat brain

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Abstract. The effects of neonatal undernutrition and postweaning protein deficiency on the content and lipid composition of gray and white matter of 63 days old rat brain have been studied. The concentrations of different lipids remain the same, but the relative proportion of gray and white matter changes thus reflecting the differences in the concentration of whole brain lipids.

Keywords. Protein deficiency; white matter; gray matter; lipids.

Introduction

Reddy and Horrocks (1982) and Reddy *et al.* (1982) have recently reported that neonatal undernutrition is associated with significant changes in the lipid composition and the contents of gray and white matter in the rate brain. Further, they have also shown that nutritional rehabilitation for 6 weeks reverses the deficits observed in gray matter whereas in the case of white matter the lipid concentrations but not the contents come back to normal (Reddy *et al.*, 1982). The present study is concerned with the effects of undernutrition during suckling period superimposed by the postweaning protein deficiency on the lipid composition as well as the proportions of gray and white matter.

Materials and methods

Albino rats of the Charles-Foster Strain inbred in our animal house for several generations were used in these studies. Pups born on the same day in the stock colony were pooled together and assigned in litters of eight to mothers fed either stock diet (18% protein, control) or a low protein diet (5% protein, undernourished). The pups were weaned at 21 days of age in both the groups. At 21 days of age the pups from control group were given a high protein diet (20%) and the pups from undernourished group received a low protein diet (4%) for 6 weeks. All the rats were caged individually and food and water were given *ad libitum* throughout the experimental period. The composition of different diets used in this study are given elsewhere (Rajalakshmi and Nakhasi, 1974, 1974a, 1975).

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At the end of the experimental period (i.e. 63 days of age), all the rats were killed, brains removed and gray and white matter separated as described previously (Reddy and Horrocks, 1982). Lipids were extracted from the separated gray and white matter by the method of Folch *et al.* (1957). The methods for the estimation of different lipids were as described previously (Reddy and Horrocks, 1982; Reddy *et al.*, 1982).

All the chemicals used in various estimations were of Analar grade.

Results and discussion

When the neonatally undernourished rats were nutritionally stressed by feeding a low protein diet during postweaning period the body and brain weights were decreased further (table 1). A decrease of 71 and 24% respectively for body and brain weights at 3 weeks òf age and 82 and 30% at 9 weeks of age were observed. Similar observations have been made by other workers (Guthrie and Brown, 1968;

Table 1. Effect of neonatal undernutrition superimposed by postweaning protein deficiency on the lipid concentrations of rat brain gray and white matter at 63 days.

	Gray matter		White matter	
	Control	Protein deficient	Control	Protein deficient
	mg/g fresh weight*			
Cholesterol	14.7±0.43[a]	14.7±0.23	28.1±0.73	28.2±0.10
Galactolipids	4.5±0.20	4.4±0.10	28.2±0.56	27.1±0.93
Phospholipids	39.1±0.42	39.9±0.65	57.0±0.82	55.4±1.10
Plasmalogens	5.0±0.21	5.0±0.14	16.8±0.19	16.3±0.50
Ganglioside NANA (µg/g)	925±26	893±38	360±6	370±9
Phosphatidyl ethanolamine	14.5±0.43	15.0±0.29	23.4±0.56	22.6±0.58
Phosphatidyl choline	15.3±0.24	15.9±0.15	17.4±0.85	17.6±0.26
Sphingomyelin	2.4±0.02	2.5±0.12	3.9±0.33	4.3±0.20
Phosphatidyl serine + phosphatidyl inositol	6.7±0.20	6.7±0.10	11.1±0.62	11.5±0.40

Body weights (g) were 179±10 and 33± 1.1 and brain weights (g) were 1.73±0.02 and 1.21±0.02 for control and protein deficient groups respectively.

Experimental details are given in the text. The mean recovery of phosphorus in these components from TLC plates was 99.5%. NANA-N-acetyl neuraminic acid.

* Represents results from 7 observations. [a] Mean ±S.E.

Fishman *et al.*, 1971; Krigman and Hogan, 1976; Reddy and Sastry, 1978). However, no significant differences were obtained between the control and protein deficient rats with regard to the concentration of different lipids either in gray matter or in white matter (table 1). It is interesting to note that the decrease

observed in the concentration of different lipids ·at 21 days of age (Reddy and Horrocks, 1982) are bridged inspite of continued nutritional stress during the postweaning period. Similar observations have been made in this laboratory with regard to glutamate decarboxylase (Rajalakshmi and Telang, 1975).

The lack of difference in the concentration of different lipids between control and protein deficient rats in both gray and white matter contrasts with the lack of similar phenomenon in the whole brain (Krigman and Hogan, 1976; Reddy and Sastry,1978).This discrepancy could prehaps be accounted for by the changes in the proportions of gray and white matter. Attempts were therefore made to estimate the gray and white matter content using the formula proposed by Reddy *et al.* (1982) namely a $X+b (1-X)-C$, where a represents the galactolipid concentration of gray matter, b, the galactolipid concentration of white matter and c, the galactolipid concentration of whole brain. The data are presented in table 2.

Table 2. Effect of neonatal undernutrition and subsequent protein deficiency on the content of rat brain gray and white matter.

	21 Day*			63 Day		
	C	UN	$\dfrac{UN \times 100}{C}$	C	PD	$\dfrac{PD \times 100}{C}$
Whole brain (mg)	1440	1100	76	1730	1210	70
Gray matter (mg)	1172	957	82	1111	926	83
White matter (mg)	264	141	53	619	288	47
% of whole brain						
Gray matter	81.6	87.2	—	64.2	76.3	—
White matter	18.4	12.8	—	35.8	23.7	—

* Values taken from Reddy *et al.* (1982)

C, Control; UN, undernourished; PD, protein deficient.

For experimental details see under materials and methods,

Values for gray and white matter were calculated as described in the text, using galactolipid concentration in whole brain, gray matter and white matter. The galactolipid values for whole brain were taken from the unpublished data of Mr. Hargit Singh, the values being 13.0 and 9.8 mg/g wet weight for control and protein deficient groups, respectively.

The decrease in the gray matter content (17%) is similar to that observed at 21 days of age. However, the increased reduction in the weight of whole brain observed at 63 days of age is reflected in greater reduction in the white matter content, the decrease being 24 and 30% respectively for the whole brain and 47 and 53% for the white matter at 21 and 63 days (table 2). The decrease in the content of gray matter correlates well with the delayed neuropil development (Cragg, 1972) and synaptogenesis (Gambetti *et al.*, 1974; Shoemaker and Bloom, 1977; Pysh *et al.*, 1979). Similarly the decreased myelin content in the undernourished rats

(Fishman *et al.*, 1971; Nakahasi, *et al.*, 1975; Wiggins and Fuller, 1978; Reddy *et al.*, 1979). The content of different lipids seems to be affected both in gray and white matter but the effects seems to be more on white matter (table 3).

Table 3.   Effect of neonatal undernutrition and postweaning protein deficiency on the content of different lipids in rat brain gray and white matter.

	Whole brain*	Gray matter	White matter
	(% of control values)		
Cholesterol	62	83	47
Galactolipids	43	82	45
Phospholipds	65	85	45
Plasmalogens	64	84	45
Gangliosides	75	80	48
Phosphatidyl ethanolamine	59	86	48
Phosphatidyl choline	64	87	47
Sphingomyelin	66	87	47
Phosphatidyl serine+ phosphatidyl inositol	63	82	48

* Values taken from Reddy and Sastry (1978).

Values for gray and white matter were calculated from the mean values given in tables 1 and 2.

In conclusion, the neonatal undernutrition superimposed by postweaning protein deficiency seems to affect the quantity of both gray and white matter. The effects seems to be more on white matter. However, the quality of gray and white matter seems to be spared with regard to lipid concentrations inspite of the continued stress during postweaning period. Thus the qualitative and quantitative differences reported between control and undernourished rat brain (Krigman and Hogen, 1976; Reddy and Sastry, 1978) seems to be due to the changes in the proportions of gray and white matter.

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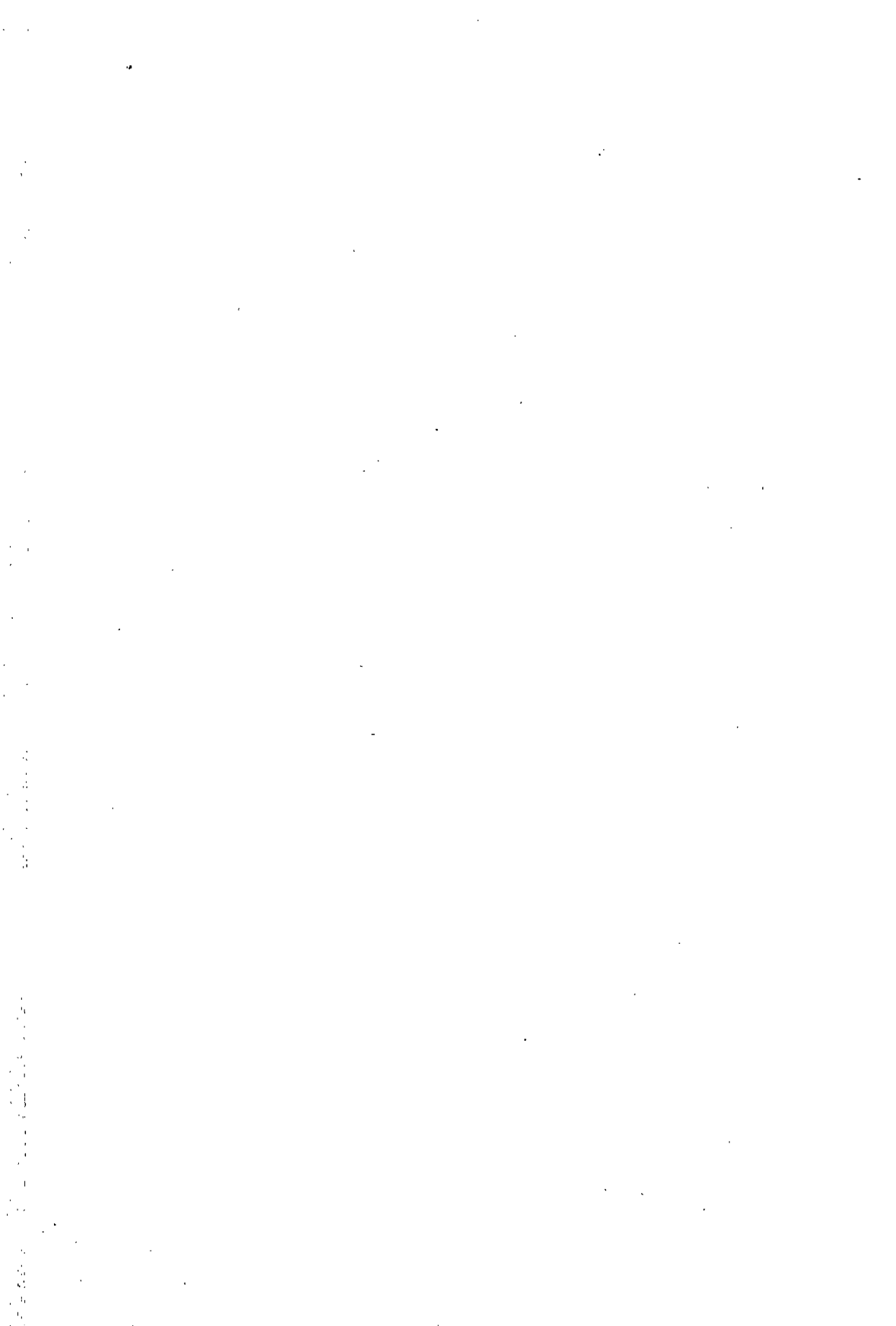
Ultrastructural features of the principal cell in the epididymis of the rhesus monkey

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Abstract.   The ultrastructural features of the principal cell in the epididymal epithelium of the monkey epididymis are suggestive of the cell carrying out a dual function of absorption and secretion. Both these functions occur on the luminal surface of the cell as well as on the lateral and basal aspects of the cell which face the intercellular spaces. Transmision Electron Microscopic studies of epididymal tissues following their impregnation with lanthanum nitrate indicated that the intercellular spaces are effectively sealed-off from the luminal space by the apically situated tight junctions between adjoining principal cells. The intercellular spaces are contiguous with the perivascular spaces of the subepithelial blood capillaries. It is suggested that the absorptive and secretory functions occuring on the apical surface of cells may be related to the creation of an appropriate intraluminal milieu for the maturation of spermatozoa while the occurrence of these functions in the intercellular spaces may represent an exchange of substances between the principal cells and the subepithelial capillaries.

Keywords.   Epididymis; rhesus monkey; ultrastructure; male reproduction; epithelium.

Introduction

Spermatozoa traversing the epididymis undergo a complex process of biochemical, physiological and morphological changes termed 'sperm maturation' which endows them with the ability to fertilize ova (Bedford, 1975). This knowledge has been the basis for considering the epididymis as one of the potential targets whose functions could be extraneously interferred with for purposes of regulating male fertility.

   Studies on non-primate species, particularly the rat, have shown that the absorptive and secretory functions of the epithelial cells lining the epididymal lumen are largely responsible for the creation of an intraluminal milieu conducive to sperm maturation (Hoffer *et al.*, 1973; Brandes, 1974; Hamilton, 1975; Wong *et al.*, 1978; Jones *et al.*, 1979). The mammalian epididiymis is known to secrete glycerophosphoryl choline, sialic acid, lipids and protein (Hamilton, 1975).The

scretion of these substances has been demonstrated *in vitro* in the principal and basal cells isolated from the epididymal epithelium (Killian and Chapman, 1980). Similar detailed studies are singularly lacking in primate species although it is fairly well-known that the epididymis in primates is structurally different in certain aspects as compared to that of the rat and other lower mammals (Alsum and Hunter, 1978; Prakash *et al.*, 1979; Ramos and Dym, 1977).

Information on the ultrastructural features of absorption and secretion of the epithelial cells in primate species would be of pertinent relevance for developing and testing such of the fertility regulating agents interfering with epididymal function. In this communication we report certain salient features of the epididymal epithelium in the rhesus monkey with particular reference to the ultrastructural correlates of absorption and secretion. A preliminary report of this study has been presented earlier (Prakash *et al.*, 1980).

Materials and methods

Tissues from the initial, middle and terminal segments of the epididymis (Prakash *et al.*, 1979), were taken from healthy adult rhesus monkeys. The tissues were fixed by vascular perfusion with or by immersion in Karnovsky's fluid containing 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Post-fixation was carried out in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at 4°C. The tissue were further processed for electron microscopy as described (Anand Kumar *et al.*, 1980). Small pieces of tissues were also processed according to the method of Shaklai and Tavassoli (1977) for tracing the contiguity of intercellular spaces by using lanthanum nitrate.

Results

General features

Principal cells are distinguished by the presence of stereocilia (figures 1, 2 and 3) and an extensive, well-developed Golgi apparatus (figure 4 and 5). Differences in the light microscopic features of principal cells situated in different segments of the epididymis have been reported earlier (Prakash *et al.*, 1979). The present ultrastructural studies have revealed that the apical surface of principal cells in the initial segment protrudes into the epidiymal lumen (figure 3). Such cytoplasmic protuberances were found only in the principal cells occuring in the initial segment. The presence of the cytoplasmic protuberances both in material fixed by immersion or by perfusion suggests that these protuberances are not fixation artifacts.

The plasma membranes of adjoining principal cells are attached at their apical end by tight junctional complexes (figure 1a). At the basal end of adjoining principal cells the plasma membranes form complex interdigitations and the intercellular spaces between the plasma membranes resemble canaliculi (figure 8).

Electron microscopic observations of tissues impregnated with lanthanum nitrate showed that the tracer had permeated throughout the intercellular spaces as well as the perivascular spaces of the subepithelial blood capillaries. Lanthanum

Figure 1. The apical part of principal cell in the initial segment is distinguished by the presence of stereocilia and the occurrence of pinocytotic invaginations in the plasma membrane (arrow). The pinocytotic invaginations of vesicles have an electron-dense coat (arrowhead) (×15,000). *Inset.* A representative electron micrograph of an unstained section from a lanthanum nitrate impregnated epididymis taken from the initial segment. The tracer has penetrated the intercellular space only as far as the *zonula occludens (arrow)* which suggests that the epididymal lumen is effectively sealed off from the intercellular space by the *zonula occludens* (×57,000).
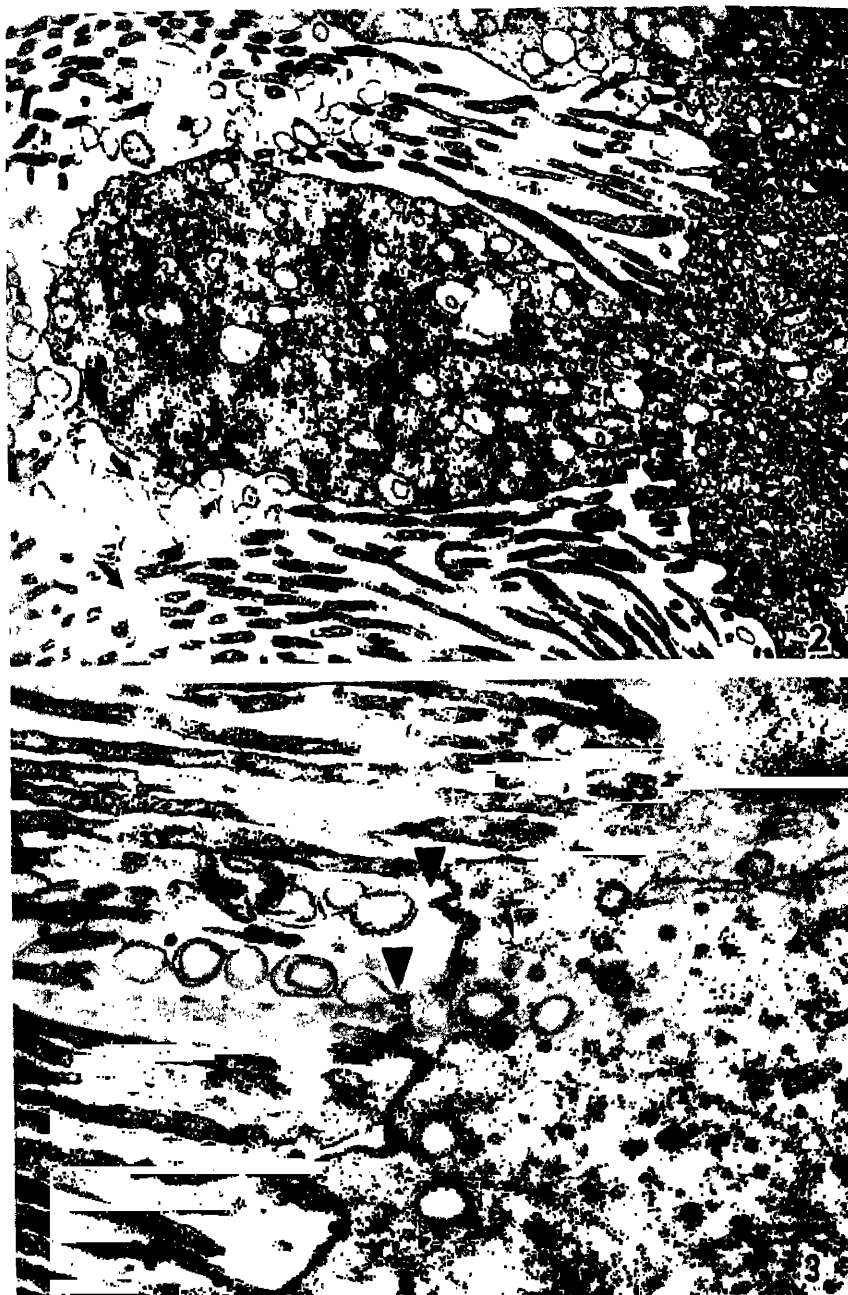
Figure 2. A cytoplasmic protuberance is seen in the apical part of a principal cell of the initial segment. The apical part of the cell includes several smooth surfaced vesicles (arrowheads) (×15,000).

Figure 3. Luminal surface of a principal cell in the middle segment of the epididymis illustrating the possible extrusion (arrowheads) of a smooth surfaced vesicle into the epididymal lumen where they appear as empty spheres as seen in figure 2 (×27,500).

Figure 4. Golgi apparatus and the associated smooth surfaced vesicle(s) in the principal cell. One such vesicle is associated (arrow) with the 'trans' face of the Golgi cistern ($\times 27,000$).

Figure 5. Electron-dense granules are also found within the Golgi appartus of the principal cell (×31,000). *Inset.* Illustrates a cluster of four secretory granules formed by the coalescence of their outer bounding membranes. This particular cluster of secretory granules is attached to the plasma membrane by a tubular stalk (*arrow*) which opens into the intercellular space. In an adjacent cell, an empty vesicle is attached to the plasma membrane. This vesicle has presumably discharged its electron-dense contents (×69,000).

nitrate did not penetrate the apically situated tight junctional complexes (figure 1a). These findings suggest that the intercellular spaces are contiguous with the perivascular spaces but are effectively sealed-off from the epididymal lumen.

The endothelial cells of the sub-epithelial blood capillaries contain many pinocytotic vesicles of the type described by Palade (1960) (figure 7).

Pinocytosis

Two types of pinocytotic invaginations were observed in the plasma membrane of principal cells. Pinocytotic invaginations having an electron-dense coat were present on the luminal, lateral and basal surfaces of the cell (figure 1, 6). These invaginations probably give rise to the coated vesicles observed within the principal cell (figure 1). The second type of pinocytotic invagination has a smooth surface and such invaginations occur mostly on the lateral and basal parts of the cell (figure 6). These invaginations perhaps give rise to the smooth-surfaced pinocytotic vesicles found within the cell.

Intracellular inclusion

Smooth surfaced vesicles containing a flocculent material and membrane-bound, electron-dense granules are amongst the distinguishing cytoplasmic inclusions to be found in the principal cell. The smooth-surface vesicles (measuring *ca.* 500nm) occur mostly in the apical region of the cell (figure 2) while the electron-dense granules (measuring *ca.* 300 nm) are concentrated in the basal part (figure 7). The ultrastructure of the Golgi region suggests that the smooth surfaced vesicles are formed on the 'trans' face (Palade 1975) of the Golgi apparatus (figure 4) while the electron-dense granules occur at the terminal ends of the Golgi cisterns (figures 4, 5).

The smooth-surfaced vesicles appear to be extruded from the apical surface of the principal cells into the epididymal lumen where they appear as hollow sphere (figure 2, 3).

The membrane-bound electron-dense granules found in the principal cells closely resemble the secretory granules found in certain endocrine organs such as the adenohypophysis or the pancreas. The granules in the principal cell occur either individually or in clusters formed by the coalescence of their bounding membranes (figure 5). Some of the electron-dense granules were found attached to the plasma membrane by tubular extensions of their surrounding membrane (figure 5a). In a fortuous plane of sectioning (figure 5a) or in section observed by tilting the goniometer stage, one can visualise the opening of the tubular extensions into the intercellular spaces. In a few instances, empty membrane-bound vesicles were also found attached to the plasma membrane (figure 5a, inset). These ultrastructural features suggest that the contents of the electron-dense granules are perhaps discharged into the intercellular space; however, the actual process of such a discharge is yet to be observed.

The ultrastructural features of the principal cell observed in several electron micrographs obtained during the present study are illustrated diagrammatically in figure 8.

Figure 6. Illustrates the basal parts (towards the right side of the picture) of two adjoining principal cells in the middle segment of the epididymis showing the interdigitation of the plasma membranes which results in the intercellular spaces appearing as a canaliculi-like strucutre (*arrow*). Smooth surfaced pinocytotic invaginations and smooth surfaced pinocytotic vesicles (*arrowheads*) occur on the canaliculi-like structure. Pinocytotic invaginations with an electron-dense coat occur on the lateral and basal surfaces of the cell (×31,500).

Figure 7. Membrane-bound, electron-dense secretory granules occur mostly towards the basal part of the principal cell (*arrow*). The endothelial cell (E) of the sub-epithelial blood capillary contains many pinocytotic vesicles some of which are found on the luminal surface of the sub-epithelial blood capillary (C) and others occur towards the perivascular space (P) (×45,000).
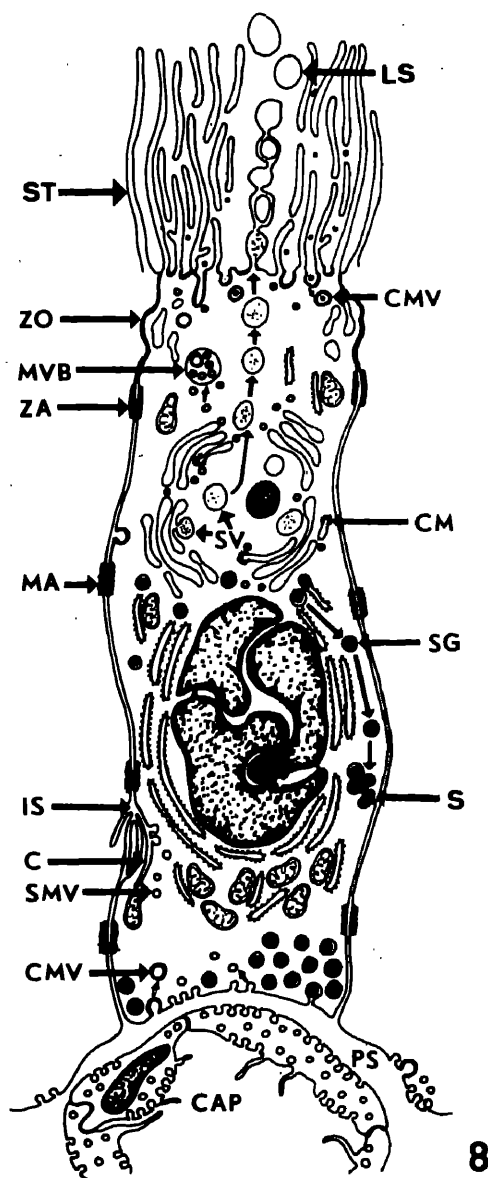
Figure 8. A composite diagram of the ultrastructural features of the principal cell as illustrated in the electron micrographs figures 1-7. The possible sequence of events related to the secretory function of the cell, as interpreted from the electron micrographs, is shown. Abbreviations: C: Canaliculi-like structures formed by the intercellular adjoining principal cells. CAP: Subepithelial blood capillary. CMV: Coated pinocytotic vesicle. CM: Coated microvesicle. IS: Intercellular space. LS: Luminal sphere. MA: *Macula adherens*. MVB: Multivesicular body. PS: Perivascular space. S: Site at which the electron-dense secretory granules are attached to the plasma membrane by a tubular stalk opening into the intercellular space. SG: Electron-dense secretory granules. SMV: Smooth surfaced pinocytotic vesicles. ST: Stereocilia. SV: Smooth surfaced secretory vesicle which are extruded into the epididymal lumen. ZA: *Zonula adherens*. ZO: *Zonula occludens*.

Discussion

The ultrastructural features of the principal cell indicate that the cell carries out a dual function of absorption and secretion. The absorptive processes are carried out by the formation of coated and smooth-surfaced pinocytotic invaginations. It is interesting to note that the coated pinocytotic invaginations are found on all aspects of the cell whereas the smooth-surfaced pinocytotic invaginations occur only at the lateral and basal regions of the principal cell. It remains to be shown whether the formation of two types of pinocytotic invaginations are indicative of the cell incorporating qualitatively different types of substances. Other studies have shown that coated pinocytotic vesicles incorporate protein-rich fluids (Roth and Porter, 1964; Friend and Farquhar, 1967) while the smooth-surfaced pinocytotic vesicles have been associated with the transcellular transport of electrolytes (Palade, 1960; Kurosumi, 1961).

The secretory products of the principal cells are formed in the Golgi apparatus and they are of two types. The smooth-surfaced vesicles are found mostly in the apical part of the cell while the electron-dense, membrane-bound granules are found more towards the basal part of the cell. Polarization of secretory products within the cell could perhaps indicate the sites at which they are secreted. The smooth-surfaced vesicles are extruded into the epididymal lumen while the products contained in the electron-dense granules are perhaps discharged into the intercellular spaces. Ultrastructural evidence indicative of secretory activity has also been reported in the initial segment of a number of mammals (Nicander and Malmqvist, 1977) and in the principal cells of vas deferens in man (Hoffer, 1976).

Ultrastructural studies of lanthanum-nitrate impregnated epididymis clearly indicate that the intercellular spaces are effectively sealed-off from the epididymal lumen by the apically situated tight junctions of the principal cells. A similar conclusion was arrived at by other investigators studying the rat (Friend and Gilula, 1972) and mice (Suzuki and Nagano, 1978) epididymis. The intercellular spaces, however, appear to be contiguous with the perivascular spaces of the sub-epithelial capillaries. The occurrence of large number of pinocytotic vesicles in the endothelial cells of the sub-epithelial capillaries strongly indicates the possibility of an exchange of substances between the intercellular spaces and the blood capillaries. Thus, it would appear that the epididymis contains two spatially separated compartments *viz.*, the luminal compartment and the intraepithelial compartment comprising the intercellular spaces.

The precise functional significance of the process of absorption and secretion carried out by the principal cells is not known. It is possible that the occurrence of both these activities on the apical surface of the princiapl cell contribute to the creation of an appropriate luminal milieu for the maturation of spermatozoa as has been suggested by other investigators (Nicander and Malmqvist, 1977; Wong *et al.*, 1978). The process of absorption and secretion occuring in the intraepithelial compartment may well represent an exchange of substances between the intercellular spaces and the subepithelial capillaries. If this is indeed so then it is most likely that the products secreted by the electron-dense granules into the

intercellular spaces may find their way into the sub-epithelial blood capillaries and they may thus have an endocrine function.

While most of the previous studies on the mammalian epididymis have focussed their attention on the absorptive and secretory activities occurring on the luminal surface of the epididymal epithelium, the present studies have drawn attention to similar activities occurring in the intraepithelial compartment. The functional significance of these activities occurring in the intraepithelial compartment needs to be further elucidated.

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Assessment of three simple techniques for the screening of circulating immune complexes: Correlation with a parameter of complement consumption

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Abstract. The sera of 36 normal controls, 45 patients with various diseases and 11 pregnant women were screened for circulating immune complexes using three relatively simple and inexpensive techniques. These included inhibition of agglutination of IgG coated latex particles with a serum having rheumatoid factor activity, polyethylene glycol precipitation and anti-complementary activity test. The circulating immune complexes were detected in a significantly higher proportion of patients as compared to normal controls. In the patients, the presence of circulating immune complexes did not always correlate with clinically detectable immunoinflammatory tissue damage indicating that pathogenic as well as nonpathogenic immune complexes were being detected by the above mentioned techniques. The alpha-1-antitrypsin/C3 ratio, however, correlated well with clinically apparent immuno-inflammation.

Keywords. Circulating immune complexes; polyethylene glycol; anti-complementary activity; alpha-1-antitrypsin/C3; aggregated human IgG; latex agglutination inhibition.

Introduction

The presence of circulating immune complexes is being shown to be important for diagnosis and prognosis in an increasing number of clinical conditions (Cochrane and Koffler, 1973; Cochrane and Dixon, 1976). A number of methods are available and are extensively used for the detection of circulating immune-complexes (Zubler and Lambert, 1977). Some of them are based upon the direct detection of immune-complexes and include both the antigen specific and antigen nonspecific methods. Other methods are indirect and dependent upon physico-chemical and/or biological properties characteristic of immune-complexes and not found in uncomplexed antigen or antibody. Because of the large number of methods available the selection of a particular technique suitable for clinical investigation of immune complexes is often difficult. Ideally, the method should be highly specific and sensitive for circulating immune complexes. Also, for the test to be clinically meaningful, it must preferentially detect the harmful pathogenic immune complexes. An additional prerequisite would be that the technique should be simple.

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The present study describes the application of 3 relatively simple techniques for detecting circulating immune complex in healthy and disease conditions. In addition, a method for detecting complement consumption dependent upon immunologically mediated (type III hypersensitivity) inflammation was also investigated. The results indicate that these four techniques in combination may be suitable for most laboratories in this country.

Materials and methods

Subjects

Thirty six normal healthy controls from amongst the faculty members, resident staff and laboratory persons with no history of any recent illness were included in the present work. Their ages ranged from 18 years to 40 years. The patient group included those with infection (upper respiratory tract infections) systemic connective tissue diseases (systemic lupus erythematosus, progressive systemic sclerosis, rheumatoid arthritis), and malignancies (leukemias and carcinoma breast). A few patients with idiopathic thrombocytopenic purpura and 11 women in first trimester of normal pregnancy were also studied.

Diagnosis was made by standard clinical and investigative parameters. Only proven cases of respective conditions were included in the study.

Collection of serum

The blood was collected in fasting state from all the subjects. The serum was kept at −20°C till tested (not more than 6 weeks). However, for anticomplementary activity, the sera were usually tested within a few days, but not later than 2 weeks after collection.

Preparation of aggregated human IgG

Standard DEAE−cellulose ion exchange chromatography was used for the purification of IgG from normal human serum (Cann *et al.*, 1971). Phosphate buffer, 0.05M, pH 8 was used for the chromatography. The purity of IgG was tested by the standard microslide technique of immunoelectrophoresis using anti-whole human serum raised in a rabbit. Purified IgG gave a single arc on immunoelectrophoresis indicating its purity. The aggregation was carried out by heating a 6 mg/ml solution of IgG at 63°C for 10 min. The aggregated human IgG was used as a positive control.

IgG-coated latex agglutination inhibition test

The technique of Lurhuma *et al.* (1976) was used. In brief, it consisted of using the serum of a seropositive rheumatoid arthritis patient with known titre of rheumatoid factor as an indicator. This was mixed with the serum to be tested for circulating immune complex and then mixed with IgG-coated particles (latex reagent). Allowance was made for dilution factors. Reduction in the capacity of rheumatoid arthrites serum (indicator) to cause agglutination of IgG-coated latex particles by the serum under test, was considered as indicative of the presence of circulating immune complex. Aggregated human IgG was used as a positive control.

Polyethylene glycol precipitation test

The technique of Haskova *et al.* (1978) was used. Two ml of borate buffer (pH 8.4, 0.1 M) and 2 ml of buffered polyethylene glycol (PEG) 6000 solution (4.16%) of PEG in borate buffer) (Sigma Chemical Co., St. Louis, Missouri, USA) were separately added to 0.22 ml of test serum pre-diluted to 1 : 3 with borate buffer (so as to obtain a final concentration of 3.75% PEG and 1 : 30 of serum). After standard mixing in a "Vortex mixer" and 60 min incubation at room temperature the difference in the light absorbance of the two samples (serum in borate buffer and serum in phosphate buffered saline) at 450 nm using 1 cm quartz cuvettes was measured with a Carl-Zeiss, spectrophotometer. Aggregated human IgG and borate buffer were used as positive and negative controls respectively.

Test for anti–complementary activity

Anticomplementary activity of the serum was assayed by the laboratory bench complement fixation test (Public Health Monograph, 1965) and by the technique of Verrier-Jones and Cumming (1977). Sheep red blood cells and hyperimmune sera raised in rabbits against sheep red blood cells were used as the hemolytic system. The optimal sensitizing dose of the hemolysin and the 50% hemolytic dose of the guinea pig complement were predetermined.

Serum stored at $-20°C$ was inactivated at $56°C$ for 30 min. Two fold dilutions of the sera were made in microtitre plates in 25 µl of buffer and 50 µl of complement containing 5 CH_{50} units was added to each well. Control wells contained buffer alone with 5, 2.5 and 1.25 CH_{50} units of the complement. Aggregated human IgG was used as a positive control. The plates were sealed with transparent non-toxic tapes and kept at $4°C$ overnight. Sensitized cells were prepared by mixing thoroughly 3% sheep red blood cells with the optimal sensitizing dose of hemolysin and incubating at $37°C$. After 15 min 50 µl of sensitized sheep red blood cells were added to each well and the plates were incubated at $37°C$ for 1 h. Colour standards (representing 0-100% lysis) were prepared and distributed in separate wells. The plates were centrifuged and the results were interpreted as follows.

Test serum which lysed all the red cells (100%) was scored as negative and where no lysis occurred (0%) as a positive indicator of anti-complement activity. The intermediary reactions, as compared with colour standards, were scored as 25%, 50% or 75%. Fifty per cent lysis was taken as the end point. The reciprocal of the highest dilution giving a 50% lysis was taken as the titre of the anti-complement activity of the serum.

Determination of alpha-1-antitrypsin/C3 ratio (α_1-AT/C3)

Principle and technique: As the estimation of serum C3 may not always indicate complement utilization, Lurhuma *et al.* (1976) suggested the determination of another acute phase reactant, viz. alpha-1-antitrypsin. If the inflammation is immunological in origin where C3 is being utilized, then the rise in C3 will be less in comparison to rise in α_1-AT. This would alter the ratio which would rise as against the ratio seen in normals or in acute inflammations not mediated immunologically. Therefore in the present study the α_1-AT/C3 ratio was

estimated. Estimation of serum C3 and α_1-AT was done by the standard single radial immunodiffusion technique of Mancini *et al.* (1965) with minor modifications, as suggested by Fahey and Mckelvey (1965). The anti serum to C3 was locally prepared (Malaviya, 1972). The anti serum to α_1-AT was obtained commercially (Meloy Lab., Springfield, Virginia, USA).

Results

Sensitivity of the tests

Latex inhibition test: Using aggregated human IgG at 1 mg/ml concentration the test gave a positive result upto a concentration of 330 µg/ml while the dilution of the test sample was 1:3.

Polyethylene glycol precipitation test

In a serial dilution test of aggregated human IgG (1 mg/ml), it was found to give a positive test upto a concentration of 40 µg.

Anti-complementary activity

Using aggregated human IgG in doubling dilution with a starting concentration of 1 mg/ml, the test was positive upto a titer of 1:64. Thus, this test was able to detect aggregated human IgG upto 1 µg per ml dilution.

Circulating immune complexes in controls

Of the 36 persons studied, 29 (80.5%) were negative by all the test while none were positive by all the tests. Of the 7 who were positive by one or 2 tests the titre was very low and in the anti-complementary activity test only 2 persons showed a titre of 1:8 (table 1).

Table 1. Circulating immune complexes in subjects studies.

Subjects (number)	All 3 tests positive	Only 1 or 2 tests positive				All tests negative
		Latex + PEG	Latex + ACA	PEG + ACA	Single test only	
Controls (36)	0	1	2	3	ACA-1	29
Collagen disease (19)	10	1	2	0	ACA-1 Latex-1	4
Idiopathic thrombocytopenia purpura (5)	1	0	1	1	ACA-1	1
Malignancy (14)	2	0	0	0	ACA-1	11
Upper respiratory infections (7)	2	2	0	0	0	
Normal pregnancy (11)	1	0	1	0	ACA-2 PEG-1	6

Latex, IgG coated latex agglutination inhibition test; ACA, anti-complementary activity, test; PEG, polyethylene glycol precipitation test.

Alpha-1 antitrypsin/C3 ratio in 36 normal controls

The determination of alpha-1 antitrypsin/C3 ratio in 36 normal controls showed a mean ratio of 0.72 with S.D. of ± 0.315. Thus, the ratio of 1.35 (mean plus 2 S.D.) was taken as the upper limit of normal ratio. Any value above it was considered as indicative of abnormal complement consumption *in vivo*.

As shown in table 2; none of the controls had any abnormality in alpha-1 antitrypsin/C3 ratio, thus showing the absence of any complement consumption.

Table 2. Correlation of circulating immune complex with α_1-AT/C3 ratio.

	α_1-AT/C3 upto 1.35	Ratio more than 1.35	P
Controls			
CIC+	7	0	<0.05
CIC−	29	0	
Collagen diseases			
CIC+	1	9	<0.001
CIC−	9	0	
I.T.P.			
CIC+	0	3	>0.1
CIC−	2	0	
Malignancy			
CIC+	2	1	>1.8
CIC−	10	1	
U.R.I.			
CIC+	0	4	>0.02
CIC−	3	0	
Pregnancy			
CIC+	5	0	0
CIC−	6	0	

CIC, Circulating immune complex; ITP, idopathic thrombocytopenic purpura; URI, upper respiratory infection; α_1-AT, alpha$_1$-antitrypsin.

Circulating immune complexes in diseases (table 1)

In collagen diseases: There were a total of 19 patients (10 with rheumatoid arthritis, 6 with systemic lupus erythematosus and 3 with progressive systemic sclerosis) in this group. Only 4 (21%) of these patients were negative by all tests. All these 4 patients were in complete remission.Of the 10 subjects positive by all tests 5 were rheumatoid arthritis, 3 were systemic lupus erythematosus and 2 were progressive systemic sclerosis. All these patients were in severe relapse. Of the 5 subjects who showed only 1 or 2 of the 3 tests positive for circulating immune complex, those with either anti-complementary activity and/or latex inhibition tests positive were in acute relapse. In contrast only one patient with positive

polyethylene glycol and latex inhibition test but negative anti-complementary activity, was in remission.

The results for the presence of circulating immune complex correlated well with the complement consumption as tested by alpha-1-antitrypsin/C3 ratio (table 2).

Circulating immune complex in other conditions: In 5 patients with idiopathic thrombocytopenic purpura, circulating immune complex was shown in 4 (80%) subjects. Again the positivity correlated well with complement consumption tested by alpha-1-antitrypsin/C3 ratio (table 2).

Very few patients with malignancy (breast cancer, 1 out of 7 and leukemia, 2 out 7) showed circulating immune complex (table 1). There was no complement utilisation detectable in these patients (table 2).

Minor illness studied included 7 patients with upper respiratory tract infection. Four of these patients showed circulating immune complex out of whom 2 patients were positive by all the 3 tests (table 1). The same 4 patients also showed significant increase in alpha-1-antitrypsin/C3 ratio, indicating complement consumption (table 2).

Eleven cases of pregnancy were also studied. Five amongst these were positive for circulating immune complex but only 1 was positive by all the 3 tests. The alpha-1-antitrypsin/C3 ratio was normal in all these subjects indicating non complement utilizing type of circulating immune complex, probably nonpathogenic in nature.

Correlation of clinical activity with circulating immune complex and alpha-1-antitrypsin/C3 ratio in patients and controls (table 3)

The presence of circulating immune complex generally correlated well with disease activity. On the other hand, elevated alpha-1-antitrypsin/C3 ratio

Table 3. Correlation of clinical condition with circulating immune complex and α_1-AT/C3 ratio.

Clinical state (number)		ACA positive alone or with other tests	PEG and/or latex only or with ACA also positive	α_1-AT/C3 ratio	
				>1.35	<1.35
Group* I (33)		21	21	30	3
Group* II (59)		11	7	2	57
	X^2	16.95	24.40	67.65	
	P	<0.001	<0.001	<0.001	

* Group I includes cases who were clinically active or in relapse or severe or, in case of carcinoma breast, preoperative cases or cases more than clinical state II. Group II includes normal healthy controls, normal pregnancy, cases of different diseases which were in remission or they were inactive or, in case of carcinoma breast, post-operative cases or early case up to clinical stage II only.

correlated even better with disease activity and severity. Patients with collagen diseases showed a trend such that whenever anti-complementary activity was present in combination or alone, invariably alpha-1-antitrypsin/C3 ratio was also elevated. This may indicate that anti complementary activity test detects pathogenic circulating immune complex more often. But it would require the examination of a large number of patients to prove it.

Discussion

The techniques chosen for the present work fulfil several of the prerequisites mentioned earlier. Thus the techniques are simple, fairly sensitive, give a satisfactory results with the time-honoured 'positive control' (i.e. aggregated human IgG), require little sophistication and do not involve expensive imported reagents. They seem to have a fair degree of correlation among themselves. Moreover, the presence of circulating immune complex in different conditions correlated with the severity of clinical state. However, this correlation was not absolute, several normal healthy controls and pregnant women also showed circulating immune complexes by one or more of the techniques used.

Using different techniques, several authors have reported varying proportions of normal individuals having circulating immune complexes, the figures being comparable to the present work (Lurhuma *et al.,* 1976; Theofilopoulos *et al.,* 1977). The possible presence of subclinical infection, presence of food antigen-antibody complexes, presence of some degree of aggregation of immunoglobulins, have all been implicated as the possible cause of circulating immune complex being detected in otherwise normal individuals.

The presence of circulating immune· complex in normal pregnancy is a controversial issue. Thus, some workers found circulating immune complex in all normal pregnancy (Lambart and Houba, 1974) while others did not find circulating immune complex in any of the pregnant women (Gleicher *et al.,* 1978). Later, McLaughlin *et al.* (1979) found circulating immune complex by the latex-inhibition technique but not by direct Clq binding. These workers reported 9 out of 12 pre-eclamptic pregnancies to be positive for circulating immune complex as against only 2 out of 12 normal pregnancies. Using the 3 tests mentioned above, the present work detected circulating immune complex in less than half of the pregnancies.

Circulating immune complexes were detected in a majority of patients with collagen vascular diseases. This is in agreement with the vast literature already available on the subject (Luthra *et al.,* 1975); Davis *et al.,* 1977; Levinsky *et al.,* 1977; Malaviya *et al.,* 1980). Similarly, circulating immune complex are well documented in upper respiratory infection (Schwenk and Baenkler, 1979) as was also seen in the present work. Similarly, the circulating immune complexes were found in a comparable proportion of ITP cases in the present study as reported by Lurhuma *et al.* (1976). However, the main discrepancy was seen in cases with leukemia and breast carcinoma. Thus, Carpentier *et al.* (1977), Hoffken *et al.*

(1978) and Theofilopoulos *et al.* (1977) have reported a high proportion of such pateints with circulating immune complex. However, the present work failed to detect circulating immune complex in a high proportion of these patients. The most likely explanation could be that the techniques used in the present work may not be sensitive enough to detect the special types of complexes found in malignancies.

In situations where circulating immune complexes are detected without obvious immuno-inflammatory tissue damage, it would be of interest to correlate its presence with a parameter indicating the presence of immune-complex mediated immunoinflammation. A simple and easy way to test it is to study the ratio of alpha 1-antitrypsin/C3. The study of this ratio showed that in obvious pathological states (e.g. active systemic lupus erythematosus, acute rheumatoid arthritis etc.) the presence of circulating immune complex correlated well with abnormally high alpha 1-antitrypsin/C3 ratio. But subject without any obvious pathological state with detectable circulating immune complex, did not show abnormally high alpha-1-antitrypsin/C3 ratio.

It would thus appear that the 3 tests chosen to detect circulating immune complexes are moderately sensitive. They were successful in detecting 'pathogenic' immune complexes in the majority of active cases of collagen diseases which as a group was considered a prototype of immune-complex disease (Gleicher *et al.*, 1978; McLaughlin *et al.*, 1979).

The findings of the present work also show that firstly, as reported from various laboratories (Report of WHO Scientific Group, 1977), circulating immune complexes are detectable in diverse clinical conditions. It was seen most often in collagen vascular diseases. However, a significant proportion of other conditions also showed circulating immune complexes. Secondly, a combination of simple techniques like latex inhibition, polyethylene glycol precipitation and anti-complementary activity, may be able to detect almost all varieties of circulating immune complexes. Thirdly, the screening for the alpha-1-antitrypsin/C3 ratio in the serum appeared to be a good indicator of immuno-inflammatory tissue damage due to circulating immune complexes.

In conclusion, it is recommended that laboratories with modest facilities, interested in clinical work related to immune complexes, may use these techniques with a fair degree of confidence. Also, for detecting pathogenic immune complexes capable of tissue damage through complement consumption (type III hyper-sensitivity) the alpha-1-AT/C3 ratio is possibly an excellent test.

Acknowledgements

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Circulating immune complexes in rodent and simian malaria

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Abstract. The circulating immune complexes have been detected in the sera of albino rats infected with *Plasmodium berghei* and rhesus monkeys infected with *P. knowlesi* by (i) quantitative cryoprecipitation assay and (ii) polyethylene glycol assay. In the rodent model, the levels of circulating immune complexes increased during infection and decreased considerably in the post-infection period. In the simian system, high levels were detected during peak parasitaemia. Polyethylene glycol precipitate obtained from the sera during acute *P. knowlesi* infection when analysed by immunoelectrophoresis was found to contain (i) monkey IgG, (ii) four other components of monkey plasma, (iii) two components of normal monkey erythrocytes and (iv) antigen(s) of *P. knowlesi.*

Introduction

Immune complexes have a well established role in causing tissue damage, and are also known to suppress the humoral and cellular immune responses of the host against various antigens (World Health Organisation, Technical Report Series, 1977). Soluble circulating malarial antigens have been demonstrated in rodent, simian and human malaria models (McGregor *et al.,* 1968; WHO, Technical Report Series, 1977). In human malaria, the antigens were shown to circulate either free or bound in immune complex forms for several weeks after clearance of parasites by chemotherapy (Houba and Williams, 1972). It has been suggested that malarial immune complexes trigger a pathogenic sequence in which other mechanisms, possibly autoimmune are involved later (Houba, 1975). The presence of circulating immune complexes (CIC) in experimental malaria in mice has been reported (June *et al.,* 1979; Contreras *et al.,* 1980). Although June *et al.,* (1979) obtained indirect evidence for the presence of specific malarial antigen and antibodies in circulating immune complexes in the sera of mice injected with *Plasmodium berghei,* further confirmation that malarial antigens are also present in

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Abbreviations used: PEG, poly ethylene glycol; PBS, phosphate buffered saline.

the circulating immune complexes is still awaited. There are no reports to date*, regarding the detection and chracterization of circulating immune complexes in rhesus monkeys infected with *P. knowlesi.*

In the present study, circulating immune complexes have been detected in the sera of albino rats infected with *P. berghei* and rhesus monkeys infected with *P. knowlesi* by (i) quantitative cryoprecipitation assay and (ii) polyethylene glycol (PEG) assay. Further, the circulating immune complexes which appear during the acute phase of *P. knowlesi* infection have been analysed for their components by immunoelectrophoresis.

Materials and methods

Animals

Male rhesus monkeys (*Macaca mulatta*) weighing 3 to 4 kg were quarantined and screened for tuberculosis. The animals were kept under quarantine in our animal house for 30 days and were tuberculin-tested before use. A natural diet of fresh fruits, vegetables, nuts and soaked pulses was given during the day. Water was provided *ad libitum.*

Male albino rats (Wistar strain) weighing 150-180 g were used in the study. Albino rabbits of both sexes weighing 1-1.5 kg were used for raising antisera. The animals were provided with a standard diet (wheat flour, ground nut, olive oil, black gram (*Phaseolus mungo*) NaCl and water *ad libitum.*

Strain of parasites

Plasmodium knowlesi W1 variant, was obained from Guy's Hospital Medical School, London, UK and cryopreserved in 30% glycerol at $-70°C$. (One volume of infected erythrocytes $+2$ volumes of 30% glycerol in 0.15 M phosphate buffered saline, pH 7.2). The frozen parasite stabilate was revived by passaging the material intravenously into a fresh rehesus monkey which was later used as a donor of parasites. This strain of *P. knowlesi* was uniformly lethal to rhesus monkeys causing death within 5 to 6 days after patency. The strain of *P. berghei* used in this study was obtained from Prof. P. C. C. Garnham, London School of Hygiene and Tropical Medicine, London, UK. This strain of *P. berghei* was not highly lethal to adult rats and most of the animals recovered from infection after reaching a maximum of about 35% parasitaemia.

Infection and collection of sera

Rhesus monkeys were infected intravenously with 1×10^4 parasitized erythrocytes collected in 0.15 M sodium citrate from a donor with ongoing infection. Albino rats were infected intraperitoneally with 1 ± 10^6 *P. berghei* infected erythrocytes. The donor animals showing 5-10% parasitaemia during the ascending phase of infection were used. The course of infection in the infected animals was monitored once a day between 10 and 11 a.m. by thin smears of blood. The smears were fixed in methanol and stained with Jaswant Singh and Bhattacharya stain as described by

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* Shepherd *et al.* (1982) have recently reported the presence of circulating immune complexes in *P. knowlesi* infected Kra and merozoite vaccinated Rhesus monkeys.

.ussel (1963). The parasitized cells were counted and expressed as number of
arasitized erythrocytes per 10,000 normal erythrocytes.

Blood for serum was collected from albino rats at 4 different phases of infection.
'hase-I — before infection; Phase-II — during the ascending phase of infection
/hen the animals showed 10 to 20% parasitaemia; Phase-III — during peak
ifection when parasitaemia was above 35% and Phase-IV — 6 days after the
nimals recovered from active infection. Blood for serum was collected from
hesus monkeys only during the first three phases of infection since *P. knowlesi* is
ighly lethal to rhesus monkeys.

Feneral schedule for raising antisera in rabbits

'en mg of antigen in Freund's complete adjuvant (Difco, USA) mixed at a ratio of
: 1 was injected in rabbits by intramuscular route on day 'O'. Second dose was
iven on 16th day with the same concentration of protein in complete Freund's
djuvant by the same route. Third dose was given on the 21st day with the same
rotein but without any adjuvant by intramuscular route. Fourth and fifth doses
/ere given on the 22nd and 23rd days with 1/5 of the protein concentration (2 mg)
sed for the first dose by intravenous route without adjuvant. Test bleeding was
one 6-7 days after the last dose and the sera were tested by gel diffusion for the
recipitin bands. Rabbits were bled on alternate days and the individual sera with
ood titres were pooled after testing.

Purified IgG from monkey was used at a concentration of 10 mg per dose to raise
ntiserum.

Inti-monkey plasma

[orm. ι monkey plasma separated from heparinized blood was diluted in a
roportion to give 10 mg/ml in normal saline and was used at 1 ml per dose per
ibbit by the above mentioned schedule for raising antisera.

Inti-P. knowlesi serum

lood from a rhesus monkey heavily infected (70-75%) with *P. knowlesi,* (mainly
:hizonts and trophozoites) were collected in 0.15 M sodium citrate and the cells
/ere washed twice with chilled normal saline. The cell pellet was then diluted with
qual volume of normal saline. This was quickly frozen and thawed thrice and 1 ml
f this material was mixed with complete Freund's adjuvant and used for one rabbit
; the first dose. The rest of the schedule was the same as mentioned above. This
ntiserum was absorbed with normal monkey erythrocytes overnight at 4°C by
iixing equal volumes of serum and packed normal monkey erythrocytes. The
:rum was separated from cells after centrifugation at 850 *g* for 15 min at 4°C.

Inti-normal monkey erythrocytes

/ashed normal monkey erythrocytes were diluted with equal volume of normal
iline and this was frozen and thawed thrice and 1 ml of this material was used per
ibbit as described earlier.

Polyethylene glycol assay (PEG assay)

The method of Haskova *et al.* (1978) was followed.

Quantitative cryoprecipitation assay

Blood was collected in a warm sterile syringe and was left for clotting at 37°C for 1-2 h in a sterile centrifuge tube. It was then centrifuged at 500 g for 20 min at room temperature (about 28-30°C) and 1 ml of the serum was kept at 4°C for 72 h. The cryoprecipitate formed was separated by centrifugation at 850 g 15 min at 4°C. The precipitate was washed twice in chilled normal saline and the final pellet was suspended in 2 ml of normal saline. The tube was then kept in a 37°C water-bath for 30 min and then centrifuged at 1300 g at room temperature. The supernatant was removed and its absorbance was monitored at 280 nm.

Polyethylene glycol precipitation of circulating immune complexes in serum for analysis

Essentially the procedure of Chia *et al.* (1979) was followed. To 4 ml of serum collected from a peak parasitaemic monkey, 4 ml of 8% PEG (MW-6000) in phosphate buffered saline (0.01 M pH 7.2) was added dropwise with constant stirring. After the addition was complete, the tube was left at room temperature for 1 h. It was then centrifuged at 1300 g for 1 h at 4°C. The resulting pellet was resuspended in 20 ml of 4% PEG and again centrifuged at the same speed. This procedure of washing with 4% PEG was repeated twice and after the last wash, the precipitate was dissolved in 2 ml of PBS 7.2 and this was used later as antigen in immunoelectrophoresis against (i) anti-monkey IgG (ii) anti-*P. knowlesi*, (iii) anti-monkey erythrocytes, and (iv) anti-monkey plasma.

Immunoelectrophoresis

The procedure of Graber and Williams (1955) was followed using 0.05 M veronal buffer pH 8.6 in 0.8% in 0.8% agarose A (Pharmacia Fine Chemicals). Solubilized PEG precipitate was subjected to electrophoresis (7.5 mA current per microscopic slide) for 90 min and the trenches were then filled with antisera and left for diffusion overnight at room temperature in a humid chamber. Washed, dried slides were then stained with 0.5% coomassie brilliant blue.

Results

Detection of circulating immune complexes in rodent and simian malaria

The levels of the immune-complexes in the sera of rats infected with *P. berghei* as detected by quantitative cryoprecipitation and PEG assay are depicted in figure 1. Significantly high levels of circulating immune-complexes could be detected in the sera of albino rats during the ascending and peak infection and levels decreased appreciably during the post-infection period. High levels of circulating immune-complexes were detected in sera of monkeys with very high infection (parasite density more than 45%) while at the ascending phase of infection when
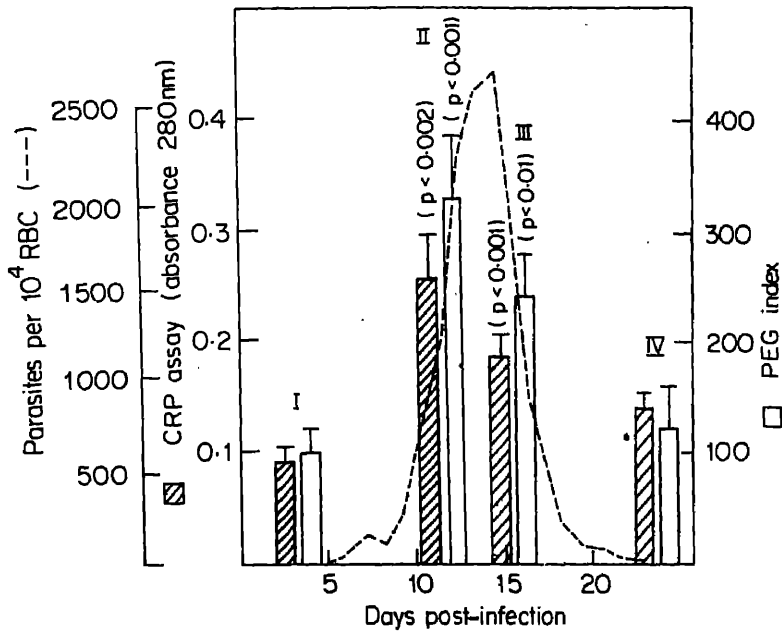
Figure 1. Content of circulating immune complexes in the sera of rats infected with *P. berghei.*

Phase-I—Control, n—16; Phase-II—10-20% infection, n—18; Phase-III—more than 35% infection, n—10 and Phase-IV—4-6 days post-infection, n—20; P values are shown only when found significant i.e. P—<0.01. The course of parasitaemia in rats has also been shown (—).

parasitaemia was between 10 and 20%, there was no appreciable increase (figure 2). The results of two tests correlated well with each other at all the stages of
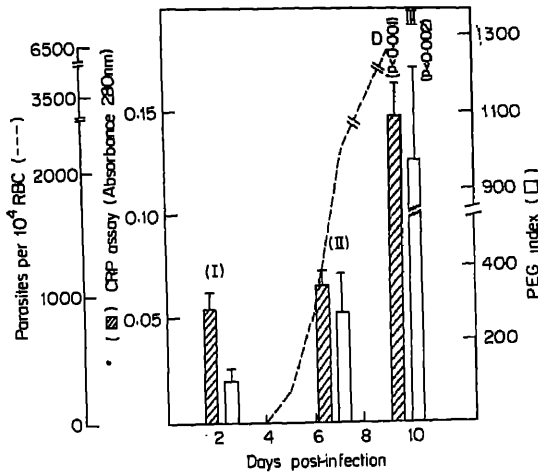
Figure 2. Content of circulating immune-complexes in the sera of rhesus monkeys infected with *P. knowlesi.* Phase-I — Control, n—14; Phase-II — 10-20% infection, n—13; Phase-III — more than 45% infection, n—14; P<0.01. The course of parasitaemia in rhesus monkeys has also been shown (—)

infection in both the rodent and the simian models, though PEG assay was found to be more sensitive than quantitative cryoprecipitation particularly in the simian model. There was a ten-fold increase in the value of PEG assay while there was only a three-fold increase by cryoprecipitation assay.

Analysis of circulating immune complexes in simian malaria

Analysis of PEG precipitate obtained from the sera of rhesus monkeys with high *P. knowlesi* infection by immunoelectrophoresis revealed the presence of monkey IgG, four other components of monkey plasma, two components of normal monkey erythrocytes, and also antigens of *P. knowlesi* origin (figure 3a to d). Solubilised cryoprecipitate obtained from *P. knowlesi* infected sera were also subjected to immunoelectrophoresis against anti-monkey IgG and anti-*P. knowlesi* serum. Similar precipitin bands were observed as recorded with the solubilized PEG precipitate.

The soluble *P. knowlesi* antigen(s) in PEG precipitate was a heat labile component getting inactivated at 56°C in 30 min or at 100°C in 5 min (figure 3e).
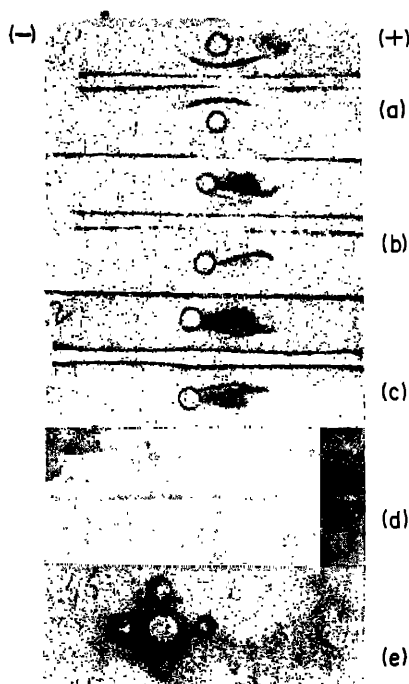
Figure 3a – d.   Immunoelectrophoresis of PEG precipitate (spotted in all the wells in a, b, c and d).

After electrophoresis the trench were filled with different antisera: a — anti monkey IgG; b — anti monkey plasma; c — anti monkey erythrocytes; and d — anti *P. knowlesi.*

Figure 3e. Gel diffusion showing heat lability of *P. knowlesi* antigen in PEG precipitate — Centre well — anti-*P. knowlesi* serum.   Wells above and below — untreated PEG precipitate. Well on the left — PEG precipitate heated at 56°C for 30 min.   Well on the right — PEG precipitate heated at 100°C for 5 min.

Although two *P. knowlesi* antigenic components could be detected by immuno-electrophoresis, only one component was visible by simple gel diffusion which was heat labile.

Discussion

The apparent normal values during the ascending phase of infection in monkeys could be due to the fact that in the simian system the infection proceeds in a logarithmic scale and reaches the peak within 5 or 6 days unlike the rodents where it takes at least 15 to 18 days to reach the zenith of parasitaemia. Although the principle behind the two tests, cryoprecipitate assay and PEG assay are different there was good correlation between the two at all the stages of infection in both the rodent and the simian system. The PEG assay however was relatively more sensitive for the monkey sera. It is essential to note that these two tests are considered relatively less sensitive as compared to a variety of other tests reported in the literature (Theofilopoulos and Dixon, 1979).

Since cryoprecipitate assay and PEG assay are antigen non-specific tests, it becomes essential to identify the components in the precipitate before attaching much significance to the values obtained in these tests.

The presence of monkey IgG has been identified by immunoelectrophoresis in both cryoprecipitate and PEG precipitate obtained from *P. knowlesi* infected sera. Facer (1980) successfully identified specific malaria antibody activity from circulating immune complexes of *P. falciparum* infected sera by indirect immuno-fluoresence test. However, in a precipitation reaction like immunoelectrophresis, immunoglobulin is more readily detected as an antigen than as an antibody. Four other plasma components were also identified in PEG precipitate using rabbit anti-monkey plasma. Although the components could not identified individually, some of them could be suspected to be complement split products. The presence of IgM, IgG, IgA and complement components in immune complexes has been demonstrated in tropical splenomegaly syndrome (Ziegler, 1973) and in human malaria (Houba *et al.*, 1976).

The demonstration of the presence of soluble *P. knowlesi* antigen(s) in the PEG precipitate and cryoprecipitate by immunoelectrophoresis is interesting. Wilson and Bartholomew (1975) had raised the possibility of circulating immune-complex associated malarial antigen to be a heat stable 'S' antigen but the malarial antigen in the PEG precipitate was shown to be heat labile, as it was inactivated in 30 min at 56°C or 5 min at 100°C.

The identification of malarial antigen in circulating immune-complexes is significant, because of the established role of such complexes in induction and effector limbs of immune response of the host against parasites (Cohen, 1976). The presence of circulating immune complexes of antigen excess have been suspected to be responsible for the chronicity encountered in many plasmodial infections (Cohen and Mitchell, 1978). Although June *et al.* (1979) showed indirect evidence for the presence of specific malarial antigen and antibodies in circulating immune

complexes in the sera of mice injected with *P. berghei,* more direct proofs were needed for further confirmation.

The demonstration of the presence of two soluble components of monkey erythrocytes in circulating immune complexes confirms the earlier observations of an autoimmune response of the host against its erythrocytes during malarial infections (reviewed by Zuckerman, 1977).

Acknowledgement

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Studies on precipitating and hemagglutinating antibodies in systemic lupus erythematosus

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Abstract. We studied the precipitating and hemagglutinating autoantibodies in the sera of patients with various connective tissue diseases in general and lupus in particular. Saline soluble extract of goat thymus had adequate antigenic materials as compared to other organs. Twenty per cent of patients with systemic lupus erythematosus were positive for precipitating autoantibodies by immunodiffusion and 44% by counterimmunoelectrophoresis. Normal human subjects, nonrheumatic disease patients and patients with rheumatoid arthritis and progressive systemic sclerosis were all negative. Forty seven per cent of positive systemic lupus erythematosus sera showed two precipitin systems. Enzyme sensitivities were used as the basis of identification of most of the antigenic specificities. Passive hemagglutination was carried out to identify antibodies to non-histone nuclear protein and nuclear ribonucleoprotein antigens. Thirty eight % of systemic lupus erythematosus patients were positive by this technique. Passive hemagglutination although a highly sensitive technique could not detect antibodies against antigenic systems other than non-histone nuclear protein and nuclear ribonucleoprotein.

Keywords. Autoantibodies; lupus; autoimmunity; rheumatic diseases; hemagglutination; precipitin reaction.

Introduction

The presence of spontaneously occurring autoantibodies to many nuclear and cytoplasmic antigens is a hall mark of diseases collectively known as autoimmune diseases which include systemic lupus erythematosus (SLE), rheumatoid arthritis, progressive systemic sclerosis (PSS), dermatomyositis, Sjögren's syndrome and mixed connective tissue disease (MCTD). A variety of autoantibodies against tissue extracts have been described in the sera of these patients (Nakamura and Tan, 1978; Talal, 1978; Steensgaard and Johansen, 1980).

Abbreviations used: SLE, Systemic lupus erythematosus; PSS, progressive systemic sclerosis; MCTD, mixed connective tissue disease; Sm, non-histone nuclear protein antigen, nRP, nuclear ribonucleoprotein; CIE, counter-immunoelectrophoresis; PHA, passive hemagglutination, GTE, goat thymus extract; PBS, phosphate buffered saline, pH 7.2; ANA, antinuclear activity.

The earliest immunological characterization of nonhistone nuclear protein which was antigenic and reactive with antibodies in human sera was reported in 1966 (Tan and Kunkel, 1966). This antigen was called Sm. Because of an extremely high selectivity of Sm antibody for SLE, it has been proposed that this might be a marker antibody (Tan *et al.*, 1978). Several other nonhistone nuclear protein antigens have been reported. One of them is nuclear ribonucleoprotein (nRNP) antigen (Northway and Tan, 1972; Reichlin and Mattioli, 1972). Antibodies to nRNP are present in very high concentration in patients with MCTD, an illness in which the symptoms represent mixtures of several types of autoimmune diseases (Sharp *et al.*, 1972).

The present work describes the studies on the precipitating and hemagglutinating autoantibodies in the sera of patients with connective tissue diseases. Most of the studies were carried out with SLE sera. The work was restricted to the study of saline soluble antigens of goat thymus which contains acidic nuclear antigens, Sm, nRNP, SS-B (Alspaugh *et al.*, 1976; Akizuki *et al.*, 1977), cytoplasmic antigens (Lamon and Bennett, 1970; Cavanagh, 1977; Koffler *et al.*, 1979), and probably others which have not been characterized so far.

Immunodiffusion and counterimmunoelectrophoresis (CIE) were used to identify the precipitating systems. Enzyme sensitivities were used as the basis of their identification. Because of the availability of small amount of sera in most of the cases, the antigenic nature of a large number of the precipitin systems could not be established fully. Passive hemagglutination (PHA) was carried out for the detection of Sm and RNP antibodies using goats thymus extract (GTE) coated formalinized sheep erythrocytes.

Materials and methods

Sera

Normal human sera was obtained from healthy individuals. Sera from non-rheumatic disease patients were obtained from various hospital out-patients. Sera of patients with SLE, rheumatoid arthritis and PSS were collected from out- and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The samples were transported to the laboratory on ice-sodium chloride mixture and stored in small aliquots at −20°C with 0.1% sodium azide as preservative. Patients with SLE, rheumatoid arthritis and PSS had features meeting preliminary criteria of the American Rheumatism Association for these disease (1973).

Chemicals

Ribonucleic acid, DNA, ribonucleas A, trypsin and agarose were purchased from Sigma Chemical Company (USA). Pronase was obtained from E. Merck (Germany). All the other chemicals were of analytical grade.

Preparation of antigen

Acetone dried powder of various organs from goat and rabbit was prepared according to the method of Horecker (1955). The reconstitution of acetone

powder was done as described by Kurata and Tan (1976). Acetone powder (60–80 mg) was mixed with 1 ml of phosphate buffered saline (PBS; pH 7.2) for 4 h at 4°C with slow continuous stirring. The resulting solution was centrifuged and supernatant stored in small aliquots at −20°C. Protein content of the supernatant was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. DNA was estimated by the method of Burton (1956) while RNA was estimated by orcinol reaction (Mejbaum, 1939).

Incubation of goat thymus extract

Goat thymus extract (GTE) was the most reactive antigen. To simulate the experimental conditions, the effect of incubation of GTE on stability of protein and RNA antigens from the extract was tested before addition of the test serum. GTE was incubated in 1 ml aliquots for different time intervals at 37°C in a thermostatic water bath upto 24 hours. The incubation was stopped by immersing the tubes in ice bath. Effect on RNA and protein content was followed after incubation by the estimation of RNA and free amino acids after each time interval.

Enzymatic digestion of GTE

The extract was treated with ribonuclease, trypsin and pronase according to the method of Kurata and Tan (1976). The ratio of enzyme to substrate by weight was 1 : 10 for ribonuclease and 1 : 50 for trypsin and pronase. Digestion was carried out for 1 h at 37°C. Appropriate controls were included in each set of experiments. The digest was centrifuged and supernatant used for further studies. RNA digestion was followed by orcinol reaction, and protein digestion by estimating free amino acids liberated (Moore and Stein, 1948).

Immunodiffusion

The precipitin reactions were performed by Ouchterlony double diffusion (Tan and Kunkel, 1966) in 0.4% agarose.

Counterimmunoelectrophoresis

CIE was carried out in 0.6% agarose using microscopic slides (Kurata and Tan, 1976).

Passive hemagglutination

Microhemagglutination test of Nakamura *et al.* (1978) with slight modification was used for the detection of antibodies to Sm and RNP antigen. GTE was used as antigen and formalinized sheep erythrocytes were employed as the passive carriers.

Results

Extraction of various tissue acetone powders with PBS resulted in preparation with a preponderance of proteins. The average ratio of proteins, RNA and DNA in different saline soluble extracts of thymus acetone powder was 4 : 0.25 : 0.01.

In immunodiffusion experiments the precipitin reactions require atleast 24 h of incubation at room temperature. It was ensured that during this period of

incubation the antigenocity of the preparations used was not affected. In order to see the effect of keeping the GTE at room temperature on the stability of protein and RNA antigens, the extracts was incubated at 37°C for different time intervals. Protein and RNA concentrations were followed after each time interval. The results indicate that there is almost complete destruction of RNA within 24 h. However, no appreciable effect on protein content was observed as indicated by a negligible increase of amino acids.

Serological characteristics of patients with various rheumatic diseases were followed by immunodiffusion, CIE and PHA.

Immunodiffusion

Using GTE as a source of antigens the precipitin reaction was negative with sera from normal human subjects and from non-rheumatic disease patients by immunodiffusion. Ten out of 50 patients (20%) with SLE showed precipitating auto-antibodies by immunodiffusion. Patients with rheumatoid arthritis and PSS were negative for precipitating autoantibodies against GTE antigens.

Counterimmunoelectrophoresis

Twenty two of 50 SLE patients (44%) had precipitating autoantibodies in their sera when the reaction was carried out by CIE using GTE as antigenic source. Normal human subjects, non-rheumatic disease patients, patients with rheumatoid arthritis and PSS were all negative.

None of the sera had precipitating autoantibodies when goat and rabbit brain acetone powder extract in PBS was used as antigen in immunodiffusion and CIE. Rabbit and goat kidney extract gave positive precipitin lines by CIE with only a few sera. The titer was considerably low as compared to GTE as antigen. Goat and rabbit heart extracts gave negative results with all the sera positive with GTE. However, liver acetone powder extracts of these animals gave similar results as with GTE.

Passive hemagglutination

Nine out of 24 SLE sera (38%) were positive by PHA with GTE coated sheep erythrocytes. The titer of the reaction ranged from 1 : 128 to 1 : 16384. Three types of reactions were observed (table 1). Three positive sera had same or almost same titer with untreated and RNase treated antigen coated erythrocytes and represented the reaction due to anti-Sm antibodies. Another three showed a considerable decrease in the titer and indicated antibodies predominantly to RNP antigen. The remaining three showed a moderate decrease in the titer and probably represented antibody activity against both Sm and RNP antigens. All the normal human sera studied had titer of less than 1 : 8.

Table 2 shows the serological findings of groups of SLE patients tested by immunodifussion and CIE. Forty seven % patients had two precipitin systems and 53% showed a single line with GTE as antigen. Out of 8 patients with two precipitin lines, four (DK, MK, RK and JS) had a weak precipitating system sensitive to RNase, trypsin and pronase, while the other system was resistant to RNase but

Table 1. Passive hemagglutination with sera of SLE patients.

Patient	Titer untreated	Titer RNase treated
PM	1 : 16384	1 : 8192
PN	1 : 128	1 : 128
JS	1 : 512	1 : 512
AS	1 : 8192	1 : 512
SH	1 : 8192	1 : 512
ST	1 : 8192	1 : 512
DK	1 : 4096	1 : 1024
MK	1 : 8192	1 : 1024
RK	1 : 2048	1 : 512

Formalinized tanned sheep erythrocytes were coated with GTE at a protein concentration of 2 mg/0.1 ml packed erythrocytes for 1 h at 37°C. RNase digestion was performed at a concentration of 1 mg RNase/0.05 ml packed antigen coated erythrocytes. One % cell suspension in 0.07% bovine serum albumin was used in agglutination reaction.

Table 2. Serological findings of some SLE patients.

Patients	Precipitin titer	No. of precipitin lines	Sensitivity of lines to			Possible identification of system(s) A/B
			RNase A/B	Trypsin A/B	Pronase A/B	
MK, RK	1 : 16−1 : 32	Two A and B	+/−	+/+	+/+	RNP/Sm
DK, JS						
AW, ST	1 : 4−1 : 8	"	+/−	+/−	+/−	RNP/SS-B
PS	1 : 16	"	−/−	+/+	+/+	?
SS	1 : 16	"	+/+	+/+	+/+	?
KR, JP PM, PN	1 : 16−1 : 32	One	−	−	−	SS-B
AS, RD	1 : 8	"	+	+	+	RNP
UR, VN, PT	1 : 8−1 : 32	"	−	+	+	Sm

Precipitin reaction was carried out by immunodiffusion and CIE using GTE as antigen. The details are given in text. Enzyme digestion was performed at 37°C for 1 h. The ratio of enzyme to substrate (w/w) was 1 : 10 for RNase and 1 : 50 for trypsin and pronase. The precipitin lines were designated as "A" and "B" for convenience.

sensitive to trypsin and pronase. These represent Sm and RNP systems respectively. The hemagglutination titer of two of these patients MK and RK was 1 : 8192 and 1 : 2048 which reduced to 1 : 1024 and 1 : 512 respectively when RNase treated antigen coated cells were used. This type of reaction is indicative of

the occurrence of antibodies to Sm and RNP antigen simultaneously. Two patients AW and ST had two precipitin systems one of which was sensitive and another resistant to all the enzyme treatments. Another patient PS showed 2 lines both of which were resistant to RNase but sensitive to trypsin and pronase.

Of nine patients with a single precipitin line, four (KR, JP, PM and PN) had system resistant to RNase, trypsin and pronase. Sera KR, JP and PM showed immunological identity by immunodiffusion (figure 1). Two sera PM and PN were
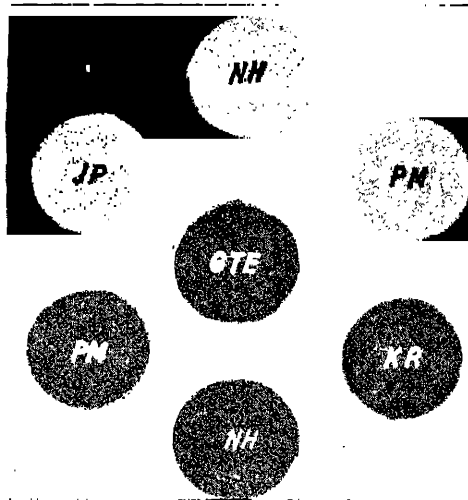
Figure 1.   Immunodiffusion reaction of SLE sera KR, JP and PM with goat thymus extract. Both KR and JP showed complete immunological identity with PM. NH represents serum from a normal individual.

positive by hemagglutination with a titer of 1 : 128 and 1 : 16384 with untreated erythrocytes and 1 : 128 and 1 : 8192 with RNase treated cells. JP and KR were negative by PHA although positive by immunodiffusion and CIE. Sera AS and RD gave a single precipitin line by immunodiffusion and CIE. The reaction was sensitive to RNase, trypsin and pronase.

Discussion

Antibodies against saline soluble mammalian antigens are of considerable clinical interest because they comprise as much as 20% of the total serum immunoglobulins in some patients with connective tissue disease (Maddison and Reichlin, 1977). These antibodies have been associated with disease subgroups with characteristic features and prognosis (Sharp *et al.*, 1972, Notman *et al.*, 1975; Farber and Bole, 1976).

Saline extracts of thymus acetone powder had adequate antigenic material reactive with antibodies from the sera of patients with SLE. This is in agreement with other studies where thymus extracts from other mammalian sources have been used for antinuclear antibody (ANA) assay (Northway and Tan, 1972; Keiser

and Weinstein, 1980). Our findings that only thymus, liver and kidney antigens reacted with SLE sera whereas antigens from heart and brain extracts were unreactive is indicative of differential distribution of various antigenic materials in the various organs of the animals. Thymus and liver are tissues which have rapid generation cycles and may probably contain many of the antigens which are absent in other organs like brain and heart which are least regenerating. This, however, does not exclude the possibility of the presence of small amounts of active antigens in these organs.

In the present study 20% of patients with SLE were positive for precipitating autoantibodies by immunodiffusion where as 44% were positive by CIE. This clearly indicates the greater sensitivity of the CIE technique over immunodiffusion. Our results are consistant with the previous report (Parker, 1973; Kurata and Tan, 1976) with respect to the sensitivity for the detection of ANA.

Using enzyme digestion studies we have demonstrated an incidence of 53% for ribonuclease sensitive system in CIE positive SLE sera which is somewhat higher than previously reported (Parker, 1973). This difference could essentially be due to combined detection of nRNP and other RNase sensitive systems including ribosomal RNP. Forty seven % of positive SLE sera had RNase resistant and trypsin sensitive systems and would probably represent Sm system although there are controversies regarding the enzyme sensitivity of this antigen (Dorsch *et al.*, 1979).

Thirty six % of our positive sera had RNase, trypsin and pronase resistant system. Whether the system represents the ill characterized SS-B or Ha or a new unknown system remains to be investigated. The association of RNase sensitive system with another system resistant to RNase probably represents the Sm-RNP whose close association has been reported by many workers (Mattioli and Reichlin, 1973; Lerner and Steitz, 1979; Waelti and Hess 1980). However, the association of RNase sensitive system with another system resistant to RNase and trypsin reported in this study is not clear. Miyawaki *et al.* (1978) described two antigens MU and TM in their studies with connective tissue disease patients. MU was sensitive to the treatment with RNase and trypsin while TM was resistant to such treatments. Later studies by Miyachi and Tan (1979) found that MU system represented rRNP. The association of RNase sensitive system with RNase and trypsin resistant system seen in present study might be rRNP equivalent of Miyachi and Tan (1979) and TM equivalent of Miyawaki *et al.* (1978).

PHA was used for distinguishing between antibodies to Sm, RNP and Sm-RNP. The technique was reproducible and almost 100 times more sensitive than precipitin reaction. An important observation in our hemagglutination reaction was that some of the SLE sera were negative for agglutination although they showed a positive precipitin reaction with immunodiffusion and CIE. Akizuki *et al.* (1977), and Keiser and Weinstein (1980) have described similar results where only S_m and RNP antigens could be detected by hemagglutination and sera positive for other extractable nuclear antigens by immunoprecipitation were

negative by PHA. Thus although a highly sensitive technique, PHA cannot be used for the detection of antinuclear antibodies other than Sm and RNP by the method described by Nakamura *et al.* (1978).

Acknowledgements

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Detection of filarial infection using *Wuchereria bancrofti* microfilariae culture antigen and filter paper blood samples in enzyme linked immunosorbent assay

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Abstract. Blood collected on filter paper by finger-prick gave results comparable to intravenous serum samples when analysed by enzyme-linked immunosorbent assay (ELISA). All the 100 microfilaraemia, 5 out of 100 endemic normals and none of the 10 nonendemic normal filter paper blood samples showed the presence of filarial antibody when tested by this method, using culture antigen and anti-immunoglubulins, class G, M and A — penicillinase conjugate. When the same samples were screened for the presence of IgM antibody, 91 out of 100 microfilaraemia, 13 out of 100 endemic normal and none of the 10 nonendemic normal samples showed a positive reaction. Enzyme linked immunosorbent assay, using culture antigen and filter paper blood samples, appears to work in large field studies for detection of filarial infection.

Keywords. Filter paper blood samples; enzyme – linked immunosorbent assay (ELISA); culture antigen.

Introduction

Blood samples collected and dried on filter paper have been shown to be useful in seroepidemiological studies for detection of parasitic diseases such as malaria (Lobel *et al.*, 1976), onchocerciasis (Ikeda *et al.*, 1978), leishmaniasis (Al-Aloust *et al.*, 1980), amoebiasis (Mathews *et al.*, 1980) and trypanosomiasis (Roffi *et al.*, 1980). These filter paper blood samples are even more relevant and will be advantageous in nocturnally periodic bancroftian filariasis, where it is difficult to collect night blood smears and in field studies. Kharat *et al.* (1982) have shown that *Wuchereria bancrofti* microfilariae culture antigen is highly sensitive in detecting filarial antibody by enzyme linked immunosorbent assay (ELISA). This study reports the use of filter paper blood samples in immunodiagnosis of human filariasis by ELISA using *W. bancrofti* microfilariae culture antigen.

Abbreviations used: ELISA, Enzyme-linked immunosorbent assay, PBS, phosphate buffer saline; BSA, bovine serum albumin; PBS/T, PBS containing 0.05% Tween 20.

Materials and methods

Sera

Venous blood was collected from filarial patients (microfilariae—positive) and normal individuals (microfilariae—negative) residing at Sevagram and its surrounding villages which is endemic for nocturnally periodic bancroftian filariasis. Serum was separated and stored at −10°C after addition of sodium azide (0.1%) as preservative. Nonendemic normal blood samples were collected from students coming to this institute from nonendemic regions such as Chandigarh, Kashmir etc. Microfilaraemia was confirmed by wet blood smear examination.

Filter paper blood specimens

Twenty µl of blood was collected by finger prick with a pipette and transferred immediately to Whatman No. 3 filter paper and was allowed to dry at room temperature. It was observed that 20 µl of blood covers a circle of diameter of about 0.9 cm. Hence the blood was collected directly on to filter paper by finger prick in such a way that it covers a circle of diameter of more than 0.9 cm. Blood on filter paper was dried at room temperature. From this a circle of 0.9cm diameter was cut for elution. Filter paper with dried blood sample was cut into small pieces and eluted into 0.75 ml of 0.5 M phosphate buffer saline (PBS), pH 7.2 into a tube by shaking at 37°C for 2 h. After centrifugation at 600 g in a clinical centrifuge for 10 min., the supernatant was separated and stored at −10°C. Sodium azide (0.1%) was used as preservative. Undiluted filter paper eluate was assigned an equivalent serum dilution of 1 : 75, assuming serum content of blood as 50%.

Enzyme linked immunosorbent assay

Penicillinase (specific activity 340 I. U./mg protein) and penicillin V were obtained from Hindustan Antibiotics, Pune. Anti-human immunoglobulin IgG+IgM+IgA as well as individual anti-human IgM (Immunodiagnostics, New Delhi), bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, USA) and polyvinyl microtitre plates (Dynatech Laboratories) were used in this study. *W. bancrofti* microfilariae culture antigen was kindly supplied by Miss Kharat of this department. The procedure for preparation of culture antigen (Kharat *et al.,* 1982) in brief, consists of separation of *W. bancrofti* microfilariae from microfilaraemia blood and maintainance for 15 days in Medium 199 (Paul, 1975) supplemented with organic acids and sugars of Grace's medium (Paul, 1975). The culture fluid collected every 24 h, was centrifuged at 13,000 g for 15 min and the supernatant was stored at −20°C and used when required. The protein in the culture fluid was estimated by Lowry's method (Lowry *et al.,* 1951).

Substrate in ELISA consisted of soluble starch (150 mg) in 27.5 ml of sodium phosphate buffer 0.2 M, pH 7.0 containing 10.64 mg of penicillin V and 65 µl of 0.08 M iodine in 3.2 M potassium iodide solution. The substrate was prepared fresh before use.

Conjugation of antihuman Ig and penicillinase was achieved by the method of Avrameas (1969) using glutaraldehyde. The optimum working dilutions of culture

antigen and penicillinase anti human Ig conjugates were determined by chequer board titration.

ELISA was carried out as described by Kharat *et al.* (1982) with some modifications. To the wells in the microtitre plate were added 100 µl of optimally diluted culture antigen (3.5 ng of protein per ml of carbonate buffer 0.06 M, pH 9.6) incubated at 37°C for 3 h and then drained. The plate was further incubated with 200 µl of 3% bovine serum albumin (BSA) in the same carbonate buffer at 37° C for 2 h. It was then washed 8 times with PBS 0.01M, pH 7.2 containing 0.05% Tween 20 (PBS/T). Hundred microlitres of test serum (1 : 300) diluted in PBS/T or same volume of equivalent blood sample dilution (in PBS/T) were added, incubated at 37°C for 3 h or overnight at 4°C, followed by washing. Then 200 µl of 3% BSA in PBS/T was added to each well, incubated at 37°C for 1 h. After washing the plate again, penicillinase antihuman IgG + IgM + IgA (1 : 50) conjugate of 100 µl volume was incubated in each well at 37°C for 3 h. After a thorough washing, the plate was incubated with 100 µl of substrate at 37°C for 1 h. The reaction was terminated by the addition of 25 µl of 5N HCl and the results were evaluated visually. The disappearance of blue colour of substrate was taken as positive reaction. The assay were done in triplicate.

Additional studies were carried out with penicillinase labelled anti human IgM (1 : 10). Kharat *et al.* (1982) observed that 3 out of 20 nonendemic normal sera gave a positive reaction at serum dilution of 1 : 160 when screened for antibody by ELISA using culture antigen and penicillinase conjugated with anti IgG + IgM + IgA. Hence we considered 1 : 300 titre as positive reaction for filarial antibody using this test system for screening of filarial sera.

Results

Twelve human blood samples belonging to microfilaraemia and control groups collected by different methods [intravenous serum (a), eluate from filter paper blood spot containing 20 µl of blood (b), and eluate from filter paper blood spot of 0.9 cm diameter (c)] were screened at the serum or equivalent blood sample dilution of 1 : 300 for the presence of filarial antibody by ELISA using culture antigen and penicillinase conjugated with IgG + IgM + IgA. Table 1 shows that the 3 types of samples i.e. a, b and c gave identical results when examined at the cut off dilution of 1 : 300. While all of the 8 microfilaraemia samples showed the presence of filarial antibody none of the 4 nonendemic normal samples were positive.

A total number of 210 human blood samples collected on filter paper belonging to different groups were screened at the equivalent serum dilution of 1 : 300 by ELISA using culture antigen and penicillinase conjugated with anti IgG + IgM + IgA and the results are summarized in table 2. All the 100 microfilaraemia and 5 out of 100 endemic normal samples tested showed the presence of filarial antibody but none of the 10 nonendemic normal samples were positive. To detect the presence of class specific IgM antibody, the same samples were screened at the equivalent serum dilution of 1 : 300 by ELISA using culture antigen and penicillinase conjugated with anti IgM. Nintyone out of 100 microfilaraemia, 13 out of 100 endemic normal and none of the 10 nonendemic samples showed the presence of

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Table 1. Comparison of sera and filter paper blood samples for detection of antibodies in ELISA using culture antigen.

Sera/blood	No. screened	Sera	No. showing positive reaction*	
			Filter paper eluate from	
			Known volume of blood (20 µl)	Known area covered by blood (circle of 0.9 cm diameter)
Non endemic normal (mf —ve)	4	0	0	0
Microfilaraemia (mf +ve)	8	8	8	8

* Sample showing positive reaction for filarial antibody at serum or equivalent serum dilution of blood sample at 1 : 300

Table 2. Analysis of microfilaraemia blood dried on filter paper by ELISA using culture antigen.

Blood	No. screened	No. showing positive reaction* with	
		anti IgG+IgM+IgA conjugate	anti IgM conjugate
Non endemic normal (mf —ve)	10	0	0
Endemic normal (mf —ve)	100	5	13
Microfilaraemia (mf +ve)	100	100	91

* Sample showing positive reaction at equivalent serum dilution of the blood sample at 1 : 300.

IgM antibody. The 5 endemic normals which showed antibody with mixed conjugate have also shown the presence of IgM antibody along with 8 more endemic normal samples.

Discussion

During sero epidemiological mass investigations of trypanosomiasis, Roffi *et al.* 1980) have found the combination of filter paper blood samples and ELISA a

valuable diagnostic tool. In filariasis, as far as we are aware, this is the first attempt to establish the utility of blood samples collected on Whatman No 3 filter paper in detection of antibody, using culture antigen in ELISA. Thus the major problem of collecting night blood samples in the field for diagnosis of filariasis can be avoided.

The diagnosis of filarial infection based on antibody detection has been frustrating because of difficulty in identifying an active infection from an exposed individual who is not having infection. In an endemic region even when soluble *W. bancrofti* microfilariae antigen was used, 55% of endemic normals showed the presence of filarial antibody (Kaliraj *et al.*, 1981). However, when *W. bancrofti* microfilariae culture antigen was used, all of the 100 microfilaraemia and only 5 of the 100 endemic normal filter paper samples showed filarial antibody with mixed conjugate. This is encouraging and agrees with observations made by Kharat *et al.* (1982) using much smaller number of serum samples.

We have observed the presence of IgM antibody in 91 out of 100 microfilaraemia cases (table 2). Lunde *et al.* (1980) have shown the presence of specific anti egg antigen IgM in all the 13 patients of early schistosomiasis thereby indicating that IgM is associated with active infection. Absence of specific anticulture antigen IgM in 9 microfilaraemia cases may be due to the transition of these cases into chronic stage where IgM antibody is mostly absent. However they did not show any early clinical manifestations when examined. Five out of the 13 endemic normals who showed the presence of IgM antibody (table 2) when further examined for microfilariae by concentration test were found to be negative. Three of these endemic normals were further examined by diethylcarbamazine (2 mg/kg body wt) provocative test and were found to be negative. These persons will be followed by periodic examination, to see whether they will become microfilaraemic in due course of time.

Denham *et al.* (1971) have shown that 30% of the microfilariae were lost at various stages of preparing and staining blood films. Southgate (1973) estimated this loss to be 50% under field conditions by experienced filariasis technicians. In the present study, by doing the wet blood smear examination in the field itself, the possibility of missing microfilaraemia cases was minimized. We still got 5 more cases out of 100 endemic normals positive by ELISA with mixed conjugate. Collection of blood samples directly on to the filter paper even in day time is an added advantage and convenient as compared to wet blood smear examination in the field at night.

Combination of filter paper blood samples and ELISA using culture antigen and mixed conjugate appears to work satisfactorily in detection of filarial infection (only 5% false positive reaction based on wet blood smear examination) in large scale field studies for undertaking drug treatment in areas endemic for bancroftian filariasis.

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The mealybug chromosome system I: Unusual methylated bases and dinucleotides in DNA of a *Planococcus* species

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Abstract. The methylation status of the nuclear DNA from a mealybug, a *Planococcus* species, has been studied. Analysis of this DNA by High Performance Liquid Chromatography and Thin Layer Chromatography revealed the presence of significant amounts of 5-methylcytosine. Since analysis of DNA methylation using the Msp I/Hpa II system showed only minor differences in susceptibility of the DNA to the two enzymes, it seemed possible that 5-methylcytosine (5mC) occurred adjacent to other nucleotides in addition to its usual position, next to guanosine. This was verified by dinucleotide analysis of DNA labelled *in vitro* by nick translation. These data show that the total amount of 5-methylcytosine in this DNA is slightly over 2.3 mol %, of which 0.61% occurs as the dinucleotide 5mCpG, 0.68% as 5mCpA, 0.59% as 5mCpT and 0.45% as 5mCpC. 5mCpG represents approximately 3.3% of all CpG dinucleotides. The experimental procedure would not have permitted the detection of 5mCp5mC, if it occurs in this system. Unusually high amounts of 6-methyladenine (approximately 4 mol %) and 7-methylguanine (approximately 2 mol %) were also detected. 6-methyladenine and 7-methylguanine occurred adjacent to all four nucleotides. The total G+C content was 33.7% as calculated from dinucleotide data and 32.9% as determined from melting profiles.

Keywords. Mealybug (*Planococcus*); chromosome imprinting; chromosome inactivation; diffuse centromere; DNA methylation; 7-methylguanine.

Introduction

Among the several unusual genetic systems found in the coccids (Coccoidea; Homoptera; Insecta), the "lecanoid" system was the first to be described (Hughes-Schrader, 1948; Brown and Nur, 1964; Brown and Chandra, 1977). This chromosome system, named after the taxonomic group in which it was discovered,

has been studied extensively in the citrus mealybug, *Planococcus citri* (Risso) (Brown and Nelson-Rees, 1961; Chandra, 1963a; Brown and Weigmann, 1969; Brown and Nur, 1964; Brown and Chandra, 1977). In *P. citri,* there are no sex chromosomes, and both males and females start development with apparently identical chromosome complements. During the early cleavage divisions of embryos which develop as males, the paternal set of chromosomes becomes genetically inactive and heterochromatic; the maternal set remains transcripition- ally active or potentially active and appears euchromatic. In embryos which develop as females no such distinction between paternal and maternal chromosome complements is seen. In adults, in the male, there is no recombina- tion during meiosis and the paternal, heterochromatic chromosomes are eliminated during meiosis II and sperm are formed only from the maternal chromosomes. Thus the maternal chromosomes of one generation become the paternal chromosomes of the next generation. In females, there is recombination, and meiosis leads to the production, as usual, of haploid eggs.

The behaviour of the paternal chromosomes in the male suggests the existence of a mechanism by which a set of chromosomes "remember" their parental origin in violation of the rules of Mendelism. The mechanism by which this impriting or "memory" is achieved is not understood. There is one other aspect of the mealybug chromosome system which is of interest from the view-point of molecular biology. This is the observation that zygotes, which are apparently genetically identical, can follow either of two developmental pathways, male or female (Nur, 1963; Chandra, 1963b; Nur, 1971). In sexually reproducing species, a strict correlation appears to exist between maleness and the inactivation or elimination of the paternal set of chromosomes. In the female, as mentioned earlier, both haploid sets function normally; there is neither inactivation nor elimination.

Inactivation or elimination of a chromosome or a whole haploid set of chromosomes when genetically similar or identical homologs in the same nucleus remains unaffected has also been described in mammals (the X chromosome) and *Sciara,* among others (Chandra and Brown, 1975; Brown and Chandra, 1977). Riggs (1975), Holliday and Pugh (1975) and Sager and Kitchin (1975) have recognized the possibility that if initiation and maintenance methylases exist in these animal systems, they would provide an attractive model for understanding such differential regulation of homologous chromosomes. Recent experiments (Liskay and Evans, 1980; Mohandas *et al.,* 1981) suggest a relationship between methylation of cytosine and the inactivation of the mammalian X-chromosome. These observations prompted us to search for modified bases in the DNA of a sexually reproducing mealybug belonging to the genus *Planococcus.*

The chromosomes of mealybugs and other coccids have an unusual centromeric organization. As first shown by Hughes-Schrader and Ris (1941), the centromeric property is not localized as in most higher organisms but distributed over the entire chromosome. As a result, chromosome fragments, whether naturally-occurring or induced, are capable of perpetuation as independent entities. By means of Cobalt- 60 irradiation of males, it has been possible to show, in *Planococcus citri,* that even

very small fragments are capable of anaphase movement during mitosis, although at a slower rate than undamaged chromosomes (Chandra, 1963a). This diffuse centromere provides an additional reason for investigation of the sequence organisation and modified bases in the DNA of this insect species.

Materials and methods

Stock cultures of mealybugs were obtained from the Horticultural Research Station, Chettahalli, Coorg, Karnataka. These mass cultures were mixtures of *P. citri* and at least one other mealybug species. Cultures of a mealybug provisionally identified as *P. lilacinus* (Cockerell) were isolated from these mass cultures and maintained on pumpkins at room temperature. The taxonomic identification was kindly provided by Dr. B. K. Rajagopal, Department of Entomology, University of Agricultural Sciences, Bangalore. Cytology of chromosome behaviour in males showed that it resembled chromosome behaviour in *P. citri* and that it was typically lecanoid (Hughes-Schrader, 1948; Brown, 1959).

DNA isolation

Nuclei were isolated from adult females, many of which were gravid. Gravid females would be expected to contain a proportion of male embryos in which the paternal set of chromosomes would be inactive and heterchromatic. Isolation of nuclei was done in the presence of citrate (Busch and Daskal, 1977) in order to prevent the formation of phenolic compounds. During initial attempts at isolating DNA from this organism, we found that due to the polyphenolic oxidases present in the haemolymph (Banks, 1976) of this insect, phenolic compounds were formed during the isolation procedure. Since these phenolic compounds bind readily and firmly to DNA, it was necessary to adopt the citrate method for isolating nuclei. The acidic conditions of the citrate method prevent the formation of phenolic compounds. Following their isolation, nuclei were suspended in saline-citrate containing 0.1% sodium lauryl sulphate, and then incubated at 37°C in the presence of proteinase K (10 µg/ml) for 1 h. DNA was extracted using buffer-saturated phenol, followed by extraction with isoamyl-alchohol:chloroform (1 : 24, v/v) and precipitation with ethanol. RNA was removed by treatment with DNase-free RNase (50 µg/ml). The DNA was re-extracted as above and precipitated with ethanol.

Digestion with Restriction enzymes

Restriction enzymes were obtained from New England Biolabs, USA. Digestion was carried out according to the conditions specified by the manufacturer. Electrophoresis of DNA was carried out in 1% agarose gels in Tris-(hydroxy-methyl)-aminomethane (Tris) (40 mM)-acetic acid (20 mM)-EDTA (2 mM) at pH 8.1. Gels were stained with ethidium bromide (5 µg/ml) for 30 min. DNA bands were visualised using ultra-violet illumination and photographed using a red filter. A 400 ASA, 35 mm negative film was used. Lambda DNA digested with Eco RI and Bam H1 was used as marker.

In vitro radio-labelling of DNA

DNA was labelled with α-^{32}P by nick translation as described by Rigby *et al.* (1977). DNA (5 µg) was nicked with pancreatic DNase I (0.5 µg/ml) for 6 min at

37°C. Nicked DNA was incubated for 10 min at 15°C in a 50 µl reaction mixture containing 50 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM cold deoxynucleotide triphosphates and 1.2 µM (250 µCi) of one of the dNTPs labelled as $\alpha\text{-}^{32}P$. Specific activities were in the range of 400 Ci/mmol to 3000 Ci/mmol in the various dNTPs, all obtained from Radiochemical Centre, Amersham, England. Incorporation of radioactivity into DNA was monitored in aliquots. After the completion of the reaction, the reaction mixture was loaded on to a diethylaminoethyl (DEAE)-cellulose column equilibrated with 0.01 M Tris-HCl (pH 7.5) and 0.1 M NaCl. After extensively washing the column with the same buffer, DNA was eluted in high salt buffer (1.5 M NaCl). The DNA was ethanol precipitated overnight at −20°C.

Dinucleotide analysis

The labelled DNA was dissolved in 16 µl of water and digested to 3′-mono-phosphates using micrococcal nuclease (140 µg/ml) and spleen phosphodiesterase (7 µg/ml) (Boehringer-Mannheim GMBH, Mannheim, W. Germany) in 100 mM Tris-HCl (pH 8.5), 10 mM $CaCl_2$ buffer. The digestion was carried out for 3 h at 37°C. An aliquot of the digest was applied to cellulose thin layer chromatography (TLC) sheets (Eastman Kodak) and chromatographed in two dimensions using isobutyric acid : water : NH_4OH (66 : 20 : 1, v/v/v) in the first dimension and saturated $(NH_4)_2SO_4$: *iso*-propanol : 1 M sodium acetate (80 : 2 : 18, v/v/v) in the second dimension (Cedar *et al.*, 1979). Chromatograms were exposed to X-OMAT (Eastman Kodak) films and autoradiograms were developed. Using these autoradiograms as templates, the respective spots from the TLC plates were scraped off and the ^{32}P radioactivity in them was measured. The spots on the autoradiograms were identified by comparison with the mobilities of standard deoxymonophosphates in the same solvent system. These standards were obtained from P.L. Biochemicals, Wisconsin, USA. Three separate isolates of DNA were studied. Each of these three samples was nick-translated and analysed in duplicate experiments.

High performance liquid chromatography

The ^{32}P-labelled 3′-monophosphates were analysed by high performance liquid chromatography (HPLC) for the presence of modified nucleotides. A 5µ Spherisorb ODS column, 15 cms long, was used. Decyltrimethyl ammonium bromide, 10 mM, in 50 mM sodium phosphate buffer, pH 6.0 was used as the eluent. The flow rate was 0.8 ml/min at a temperature of 23 ± 1°C. The eluent was monitored at 260 nm using a Schoefel-770 variable wavelength detector. The standard monophosphates (P.L. Biochemicals) were the same as those used for TLC. After the elution times of the standards had been determined, 20 µl of the sample was injected along with 50 µl of the non-radioactive, standard deoxy-nucleotides. The fractions corresponding to the known elution times of the standards were collected and their radioactivity measured.

HPLC was also performed on acid hydrolysed unlabelled DNA. A C_{18}µ-Bondapak reverse phase column was used on a Waters instrument (Model ALC-

GPC-244). The solvent used was water: methanol: acetic acid (96:4:0.25, v/v/v). The eluent was monitored at 254 nm.

Calculation of tetranucleotide frequencies

From the observed frequencies of the various dinucleotides, the probable frequencies of all possible tetra-nucleotides were calculated on a computer. The programme generated tetranucleotides as a product of the frequencies of overlapping dinucleotides within each tetranucleotide. For example, the frequency of CCGG would be the product of the experimentally observed frequencies of CC, CG and GG. Similarly, the frequency of CCCC was calculated by multiplying three times the observed frequency of CC.

Results

Consistently good preparations of DNA were obtained by use of the method mentioned earlier. Each sample of DNA was characterized spectrophotometrically as well as electrophoretically. The DNA preparations exhibited a sharp and smooth melting profile, with a Tm of 83.4°C (figure 1). The G+C content was calculated to be 33.7% on this basis (Mandel and Marmur, 1968).
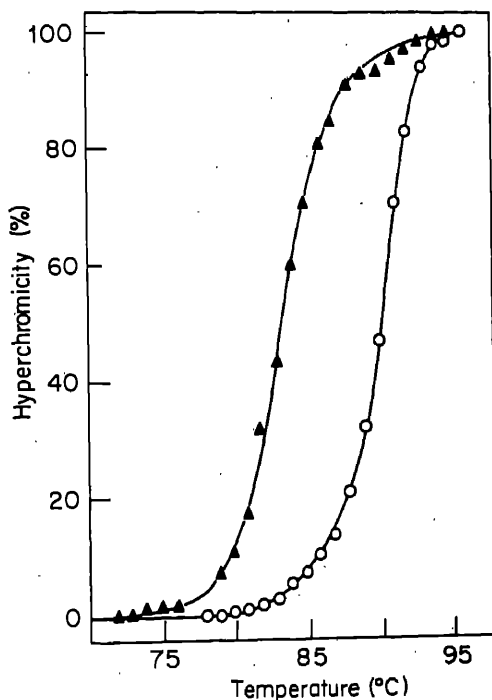
Figure 1. Thermal denaturation of DNA. Native DNA in 0.12 M sodium phosphate buffer, pH 6.8, was melted at 1°C increments in a Gilford Spectrophotometer 250 equipped with a thermoprogrammer (model 2527). *Escherichia coli* DNA was used as standard. The DNA melting temperature (Tm) and G+C content were calculated according to Mandel and Marmur (1968). The open circles represent *E. coli* DNA and the closed triangles, DNA from mealybug (gravid females).

    The restriction enzymes Msp I and Hpa II both recognize the sequence CCGG
and are useful for studying the methylation status of the CpG dinucleotide. While
Msp I cuts this sequence whether or not the internal C is methylated, Hpa II will cut
it only if the internal C is unmethylated. There was a small but recognizable
difference in the susceptibility of mealybug DNA to these two enzymes (figure 2,
lanes 2 and 3). When the DNA was digested by a mixture of the two enzymes
(figure 2, lane 4), the extent of digestion was similar to that observed with Msp I
alone. These results suggested that a small proportion of the CCGG sequences in
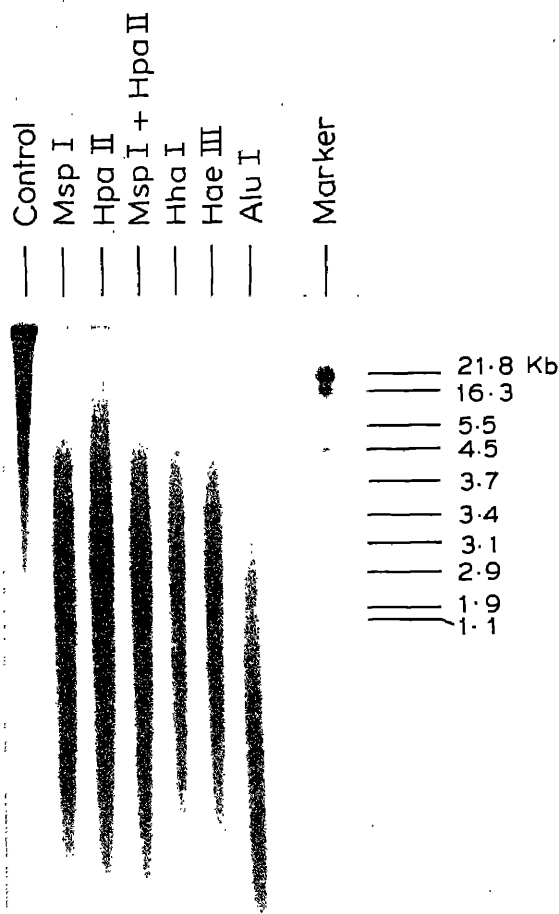
Figure 2. Restriction enzyme digestion of DNA. Two μg of mealybug DNA was digested
for 6 h at 37°C with 4 units of the enzyme. DNA fragments were separated by electrophoresis
at 50 volts for 8 h on 1% agarose gel. λ DNA digested with EcoR1 and Bam H1 was used as
molecular weight marker.

this DNA was resistant to digestion by Hpa II because the internal C was methylated. On the other hand, when bases prepared by perchloric acid digestion of DNA were analysed by HPLC (MCH 10 C-18 Micropack RP column, Varian LC 5000), large amounts of 5-methylcytosine and 6-methyladenosine (6mA) were detected (data not shown). In view of the apparently high content of 5mC in this DNA, one would expect significant differences in the susceptibility of this DNA to the enzymes Msp I and Hpa II. However, as seen from figure 2, there is only a small difference in the susceptibility of this DNA to the two enzymes, suggesting that only a fraction of the total 5mC is present in CCGG sequences. It therefore seemed likely that even if 5mC occurred in the dinucleotide CpG it was predominantly in sequences other than CCGG or that it occurred in other dinucleotides as well.

Dinucleotide analysis of labelled DNA was done to identify the dinucleotides with 5mC. The results are summarised in table 1. The two-dimensional TLC resolved, in addition to the four usual 3'-deoxy mononucleotides, d6mAMP and d7mGMP (figure 3 and figures 4a and b). On extended autoradiography, additional unidentified spots of minor components could also be visualised (figure 4c).

Following HPLC, the four normal deoxynucleotide monophosphates as well as 5mCMP and 7mGMP, are well resolved under the solvent conditions used (figure 5a). 5mCMP and 7mGMP together with the four normal deoxynucleotide monophosphates were mixed with the hydrolysate of the labelled mealybug DNA and subjected to HPLC analysis. The fractions corresponding to the known elution times of the standards were collected and radioactivity in them was counted (figure 5b). The presence of significant amounts of radioactivity in the HPLC fractions corresponding to the elution times of the standard 5mCMP and 7mGMP was taken as qualitative evidence for the presence of these modified nucleotides in mealybug DNA. Since there was more than one unidentified peak in the HPLC profiles, these data were not considered sufficient for accurately determining the extent of methylation.

HPLC of perchloric acid hydrolysate of unlabelled mealybug DNA provided additional evidence for the presence of 7mG (figures 6a, b). The identity of the 7mG peak was confirmed by peak enhancement.

As shown in table 1, 5mC in mealybug DNA occurs as extensively in CpA and CpT dinucleotides as in CpG. 5mCpC occurs to a lesser extent. 6mA and 7mG were found as 5' neighbours of A, T, C and G. Although dinucleotide analysis was done twice each on three separate samples of DNA, the quantitative data reported in table 1 may be subject to some error, for the following reason. Since the modified bases frequently moved close to their respective unmodified bases, there would be chances of cross contamination when such spots are scraped off for radioactivity measurements. As a result, accurate data about the relative proportions of the modified and unmodified bases and their relationship, if any, to the sex and stage of development of the insect should await careful HPLC analysis of the DNA of this species. Further studies along these lines are in progress.
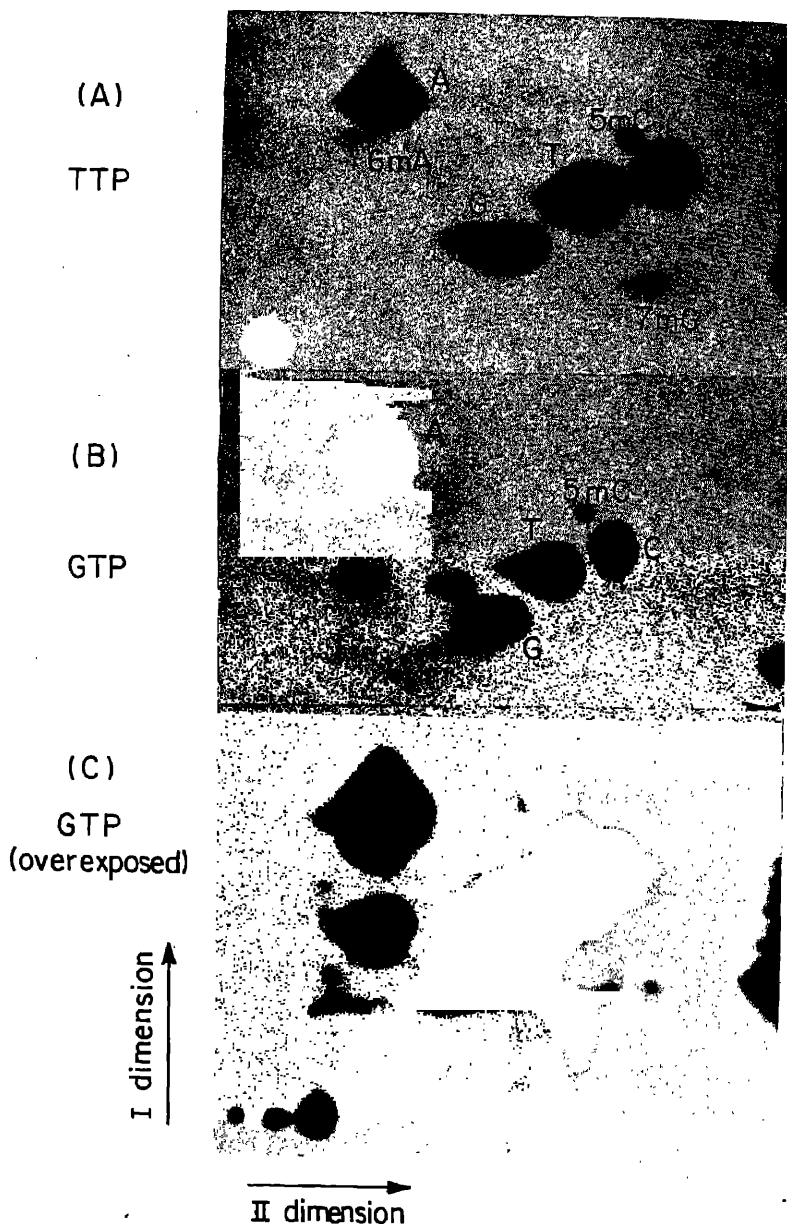
Figure 3. Dinucleotide analysis. $3'$ α-^{32}P labelled nucleotide monophosphates were chromatographed under the same conditions as those employed for separation of the standard dNMPs (figure 4).

A. Autoradiogram of $3'$ dNMPs following nick translation in the presence of α-^{32}P dTTP.

B. Autoradiogram of $3'$-dNMPs following nick translation in the presence of α-^{32}P dGTP.

C. Overexposed autoradiogram of $3'$-dNMPs following nick translation in the presence of α-^{32}P dGTP.
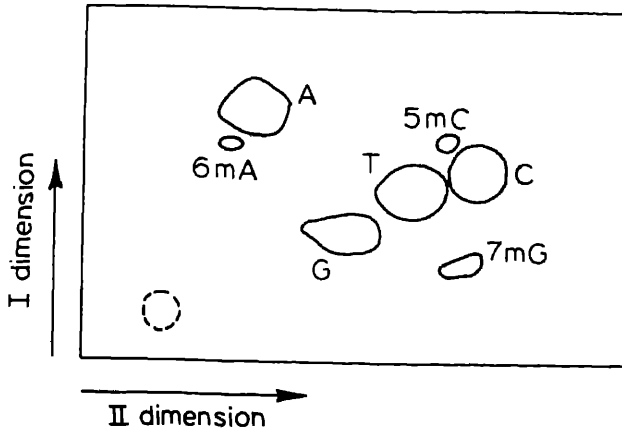
Figure 4. Separation of deoxynucleotide monophosphates on two dimensional TLC. Diagrammatic representation of observed separation of standard dNMPs following chromatography on cellulose TLC plates using Isobutyric acid : water : NH$_4$OH (66 : 20 : 1, v/v/v) in the first dimension and saturated (NH$_4$)$_2$SO$_4$: in the second dimension.
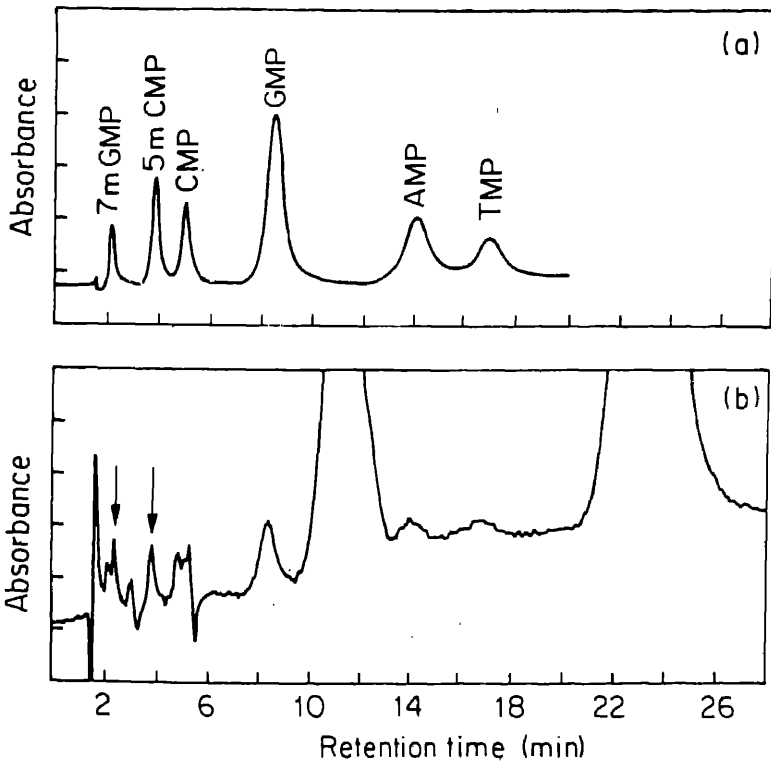
Figure 5. HPLC of deoxynucleotide monophsophates. The solvent conditions and other experimental details were as described under Materials and methods.

A. Separation of standards.

B. HPLC profile of ^{32}P-deoxynucleotide monophsphates obtained after nick translation of mealybug DNA. The two arrows indicate the peaks corresponding to 7mG and 5mC.
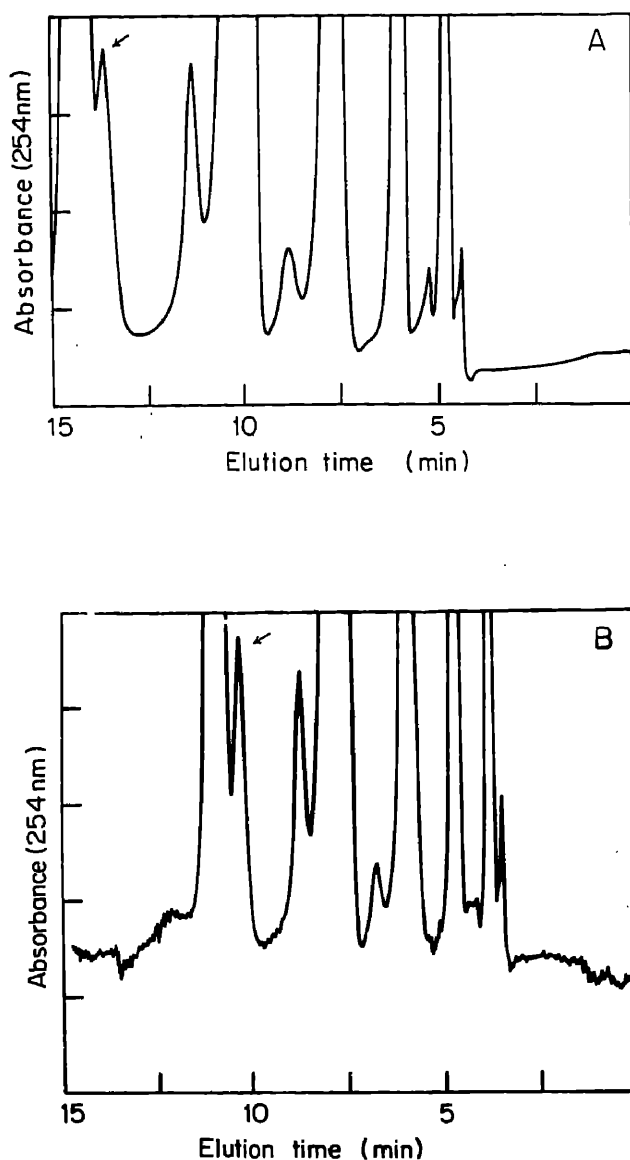
Figure 6. HPLC profiles of acid hydrolysed unlabelled mealybug DNA.

A. Separation of bases from mealybug DNA on a C$_{18}$μ-Bondapak column at a flow rate of 0.61 ml per minute.

B. Peak enhancement observed following injection of authentic sample of 7 mG along with hydrolysate of mealybug DNA. The flow rate was 0.81 ml per minute. Arrows in the two panels indicate the 7 mG peaks.

Table 1. Nearest neighbour anlysis of methylated bases in mealybug DNA.

	5′ end				
	5mC	6mA	7mG		
	0.68	0.67	0.34	A	
	0.59	0.61	0.92	T	3′ end
	0.61	0.92	0.37	G	
	0.45	1.81	0.55	C	
Total	2.33	4.01	2.18		

The G+C content calculated from the dinucleotide analyses was 32.9%, which is in agreement with the estimate from DNA melting profiles (33.7%). Of the total cytosine, 13.5% occurred as 5mC. Further, 12.5% of total adenine occurred as 6mA and 12.8% of guanine as 7mG. The frequencies of the various dinucleotides were used to compute tetranucleotide frequencies. The tetranucleotides of interest and their estimated frequencies are given in table 2.

Table 2. Calculated frequencies of certain tetranucleotides which are recognized by restriction enzymes.

DNA sequence	Frequency of the tetranucleotide, in per cent*	Enzyme recognizing the sequence
CCGG	0.44	Msp I
C5mCGG	0.07	Msp I
CCGG	0.44	Hpa II
GCGC	0.57	Hha I
GGCC	0.57	Hae III
AGCT	0.61	Alu I
GATC	1.72	Sau 3A
G6mATC	0.10	Sau 3A

* These numbers were derived from dinucleotide data. For details, see Materials and methods.

Whereas the restriction enzymes Msp I and Hpa II are useful for characterizing methylation in CCGG sequences, other enzymes such as Hha I, Hae III and Alu I are useful for studying the methylation of CpG in other tetranucleotide sequences. As seen in table 2, the recognition sequences for Hha I, Hae III and Alu I all

occurred in nearly equal proportions. From these calculated frequencies one would expect that mealybug DNA would be equally susceptible to digestion by these three enzymes. However, as seen in figure 2, (lanes, 5, 6 and 7) digestion with Alu I yielded much smaller fragments than digestion with either Hha I or Hae III. One explanation for this observation could be that the recognition sequences for the enzymes Hha I and Hae III are sometimes methylated in mealybug DNA.

Discussion

Loewus *et al.* (1964) reported that they did not detect any 5mC by paper chromatography of acid-hydrolysed DNA isolated from a mealybug, *Planococcus citri* (Risso), which belongs to the same genus as *P. lilacinus.* Nor did their data on buoyant density and melting profiles of DNA from *P. citri* suggest differences between DNAs of males and females in the content of any modified base. Subsequently, Rao and Chandra reported, in abstract form (1980a, b), significant amounts of 5mC in *P. citri* DNA as well as large differences between the two sexes in the amount of 5mC. They also reported finding, in DNA isolated from males, two buoyant density peaks whereas only one such peak was observed in DNA isolated from virgin females. It now appears that the results of Rao and Chandra (1980a, b) will have to be reassessed because the procedure adopted for isolation of DNA did not take into account the presence of polyphenolic oxidases (Wyatt, 1961) in mealybugs. If care is not taken, phenolic compounds would be formed during the isolation procedure and these would bind firmly to DNA. Since many of these phenolic compounds contain methyl groups (Banks, 1976), they could introduce artefacts during TLC and other methods of estimation of DNA methylation.

There is one other complexity of the mealybug system which is relevant to the results reported in this paper. This pertains to the presence of certain yeast–like symbionts in both sexes. They are transmitted by the mother to the egg. The symbionts invade certain polyploid cells which form a small organ called the mycetome whose function is not known. When DNA is isolated from whole animals, the possibility of "contamination" of mealybug DNA by symbiont DNA cannot be avoided. This may become important, particularly when one is studying differences between DNAs isolated from males and females, because the two sexes are vastly different in size. *P. lilacinus* appears to contain far fewer symbionts than *P. citri.* At least in the stock we are working with, it is often difficult to demonstrate symbionts in adult females, whether virgin or gravid. In addition, care was taken during this investigation to isolate DNA from purified preparations of nuclei and not directly from whole animals. As a result, we believe that contamination of mealybug DNA by symbiont DNA, if it had occurred, was negligible.

DNAs from a wide variety of organisms have been shown to contain 5mC (Ehrlich and Wang, 1981). Rather large amounts of 5mC have been reported in DNAs of certain plants whereas insects generally contain low amounts or, as in the case of Drosophila, apparently none at all. 6-Methyladenine has been reported to occur in several lower eukaryotes such as the protozoa, and in algae such as *Chlamydomonas.* It has also been reported in DNA of mosquito cells in culture. 7mG has been detected in DNA of the Shigella phage DDVI (Nikolskaya *et al.,*

976). We are aware of only one report on the occurrence of this rare modified nucleotide in eukaryotes (Culp *et al.*, 1970). The presence of very small amounts of 7mG and two other modified forms of G were reported by Culp *et al.* (1970) in DNA isolated from HeLa cells.

In the DNA of *P. lilacinus*, high levels of C methylation (5mC representing 3.5% of total C), A methylation (6mA representing 12.5% of total A) and of the unusual modification of guanine (7mG representing 12.8% of G) were observed. In other systems, 5-methylcytosine has been reported to occur most often in the dinucleotide CpG. In the data reported here, it occurs in CpA and CpT almost as often as in CpG. The frequency of its occurrence in CpC is slightly lower. The technique of dinucleotide analysis would not have permitted the detection of 5mCp5mC if it occurs in mealybug DNA (table 1). Occurrence of the unusual methylated dinucleotides 5mCpA, 5mCpT and 5mCpC has been reported in wheat-germ DNA (Gruenbaum *et al.*, 1981). However, in wheat-germ, the most commonly seen modified dinucleotide is still 5mCpG which represents 82% of all CpG dinucleotides; 5mCpT and 5mCpA occur in far lower amounts, 19% each, of the respective dinucleotide populations.

In the mealybug data reported here, 5mCpA and 5mCpT occur in high frequencies and in about equal proportions. It is therefore likely that, as in the case of wheat-germ DNA (Grauenbaum *et al.*, 1981), C methylation often occurs in the dinucleotide CXG, where X is either A or T.

As seen in table 2, the estimated frequency of CCGG sequences, which are the recognition and cleavage sites for the enzyme Hpa II, is 0.44% of all tetra-nucleotides. Msp I recognizes and cuts the above sequence both when it is unmethylated as well as when the internal C is methylated. These sites are estimated to occur at a frequency of 0.51% of all tetranucleotides. It follows from these data that the proportion of Hpa II resistant sites in this DNA would be approximately 0.07%. This would mean that only about 14% of the total CCGG sequences have a methylated internal C. This is consistent with the observation that the difference in susceptibility of this DNA to Msp I and Hpa II is very small (figure 2, lanes 2 and 3). Further, while 6mA occurs in all possible dinucleotides, it occurs most often as 6mApC.

The occurrence of high levels of 6mA, 7mG and 5mC, and their presence in a variety of dinucleotide combinations, may prove to be of interest in further analysis of this unusual chromosome system.

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ERRATA

1. Sequential release of cellulose enzymes during germination of *Trichoderma reesei* spores

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Volume No. 4, Number 3, p. 281.

i. The title of the paper should read as:

Sequential release of cellulase enzymes during germination of *Trichoderma reesei* spores

ii. In **Introduction** the line number 3 should read as:

EC.3.2.1.-) β-1,4 endoglucanase (CMCase, EC.3.2.1.4 and β-1,4 glucosidase (cellobiase, EC.3.2.1.21) ......

2. Role of *S* gene product of bacteriophage lambda in host cell lysis

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Volume No. 4, Number 3, page 365:

	Printed	To be read as
Table 1, line 1	594 (λcI ts S^- R^-)	594 (λcI ts S^- R^+
Line 6 from bottom	$S^- R^-$	$S^- R^+$
Line 3 from bottom	$S^- R^-$	$S^- R^+$

3. Production of antibodies specific to human chorionic gonadotropin in mice immunized against its chemical analogs

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Volume No. 4, Number 3, page 369, the second author's name should read as — OM P. BAHL.

4. Dosage compensation and sex determination in *Drosophila:* mechanism of measurement of the X/A ratio†

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Volume No. 4, Number 3, page 389, para 2, the last line should read as: interaction of *mle* with Sxl^{F1}.

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